The development of molecular techniques for microbial population analysis in landfills

Thesis

UNRESTRICTED

THE DEVELOPMENT OF MOLECULAR TECHNIQUES FOR MICROBIAL POPULATION ANALYSIS IN LANDFILLS

By

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ABSTRACT

Methanogens are responsible for the production of methane, which is a major component of landfill gas. Methane is a greenhouse gas, but also a potentially valuable energy source. A better understanding of the methanogens in landfill could aid the development of improved strategies for the control of landfill degradation processes.

Excavated refuse and leachate samples were obtained from five landfills. DNA was extracted from the samples and a methanogen-specific gene (*mcrA*) was amplified by PCR. Clone libraries were generated, and screened by PCR-RFLP. This revealed a much greater diversity of methanogens in landfill than had been detected in previous studies. Furthermore, the composition of the methanogen communities was substantially different between landfills, and within landfills. DNA sequencing and phylogenetic analysis was used to determine the phylogenetic affiliations of landfill methanogens. Members of the order *Methanomicrobiales* were found to be dominant in the majority of the samples. *Methanobacteriales* was the second most abundant group, while *Methanosarcinales* appeared to be only a minor component of the methanogen population in most of the samples. Phylogenetic analyses revealed five clusters of *mcrA* sequences that were not closely affiliated to any described species included in the analysis, including one cluster that was not closely affiliated to any of the five methanogen orders. These unidentified clusters may represent novel methanogenic lineages. A set of nested, group-specific oligonucleotide probes for *mcrA* was designed to detect the groups identified by the phylogenetic analysis. The usefulness of these probes for rapidly characterising methanogen communities was demonstrated by screening clone libraries of *mcrA* PCR products.

The potential of molecular techniques for detecting homoacetogenic *Bacteria* in landfill was demonstrated by the generation of PCR products from DNA extracted from landfill using primers for the FTHFS gene, and by detection of a PCR product from landfill with a probe for FTHFS.

The results of this study have greatly increased our knowledge of the methanogen community in landfill, and the molecular techniques developed in this study should prove valuable for further investigations of the methanogen population in landfill and other environments.

ABBREVIATIONS

$\Delta G^{\circ\prime}$	Gibbs free energy change
16S rDNA	Gene encoding the 16S rRNA molecule
16S rRNA	the 16S molecule of ribosomal RNA
AODC	acridine orange direct counts
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
BOD	biological oxygen demand
CAMR	Centre for Applied Microbiology & Research
CDGE	constant denaturing gel electrophoresis
cDNA	molecule resulting from reverse transcription of RNA
COD	chemical oxygen demand
CSPD	3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddUTP	di-deoxy uracil triphosphate
DES	DNA elution solution
DETR	the Department of the Environment, Transport and the Regions
DGGE	denaturing gradient gel electrophoresis
dGTP	deoxyguanosine triphosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DSM	Deutsche Sammlung von Mikroorganismen
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
dTTP	deoxythymidine triphosphate
EDTA	ethylene diamine tetra acetic acid
EfW	energy from waste
ELISA	enzyme-linked immunosorbent assay
EU	European Union
FISH	fluorescent in situ hybridisation
FTHFS	formyltetrahydrofolate synthetase
HPLC	high performance liquid chromatography
LB	Luria-Bertani
LMW-rRNA	low molecular weight-rRNA
mcrA	gene encoding subunit A of methyl coenzyme M reductase I
MPN	most probable number
mRNA	messenger RNA
*mrt*A	gene encoding subunit A of methyl coenzyme M reductase II
MSW	municipal solid waste
NA	nucleic acid
OCM	Oregon collection of methanogens
OTU	operational taxonomic unit
PAGE	polyacrylamide gel electrophoresis
PAHs	polyaromatic hydrocarbons
PCBs	polychlorinated biphenyls
PCR	polymerase chain reaction
PCR-SSCP	PCR-single strand conformational polymorphism

PPS	protein precipitation solution
Q-PCR	quantitative-PCR
RDB	ribosomal database project
RDF	refuse derived fuel
RFLP	restriction fragment length polymorphism
RFP	restriction fragment pattern
RNA	ribonucleic acid
rRNA	ribosomal RNA
RSGP	reverse sample genome probing
RT-PCR	reverse transcriptase-PCR
SDS	sodium dodecyl sulphate
SEWS-M	salt ethanol wash solution - M
sp	species (singular)
spp	species (plural)
SRB	sulfate-reducing bacteria
SSC	standard sodium citrate
SSU	small subunit
T_{opt}	optimum temperature
TAE	tris-acetate EDTA
TBE	tris-borate EDTA
TE	tris-EDTA
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
temp	temperature
TGGE	temperature gradient gel electrophoresis
TNA	*Taq* nuclease assay
TTGE	temporal temperature gradient electrophoresis
UK	United Kingdom
UV	ultra violet
VFAs	volatile fatty acids
X-Gal	5-bromo-4chloro-3indolyl-β-D-galactoside

Standard abbreviations for bases

A	adenine	M	C or A (amino)
C	cytosine	S	G or C (strong)
G	guanine	W	A or T (weak)
T	thymine	B	C, G or T (not A)
R	A or G (purine)	D	A, G or T (not C)
Y	C or T (pyrimidine)	H	A, C or T (not G)
K	G or T (keto)	V	A, C or G (not T)
		N	A, C, G or T (any)

Single letter amino acid code

A	alanine	N	asparagine
B	aspartate or asparagine	P	proline
C	cystine	Q	glutamine
D	aspartate	R	arginine
E	glutamate	S	serine
F	phenylalanine	T	threonine
G	glycine	U	selenocysteine
H	histidine	V	valine
I	isoleucine	W	tryptophan
K	lysine	Y	tyrosine
L	leucine	Z	glutamate or glutamine
M	methionine	X	any

Units

µg	microgram
µl	microlitre
ρmol	picomole
bp	base pair
°C	degrees Celsius
cfu	colony forming unit
cm	centimetre
g	gram
hr	hour
kg	kilogram
kJ	kilojoule
L	litre
m	metre
M	moles per litre
ml	millilitre
mM	millimoles per litre
mol	mole
Pa	Pascal
rpm	revolutions per minute
sec	second
V	volts
v/v	volume per volume
w/v	weight per volume
wt	weight

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1 INTRODUCTION

1.1 Overview

Landfill is the most common and widespread means of waste disposal in the United Kingdom (UK) and throughout the world. Twenty-eight million tonnes of municipal solid waste (MSW) was produced in England and Wales in 1998/99 (Anon, 2000e). Landfill was the disposal route for 83% of this waste. The activity of microorganisms in breaking down the organic portion of landfilled wastes, results in the production of landfill gas and leachate, plus a reduction in waste volume. A better understanding of the microbial communities and processes involved would allow the development of improved strategies for the control of landfills (Lawson, 1989b).

The analysis of microbial communities in the environment has traditionally been reliant on cultivation of the microorganisms, many of which are notoriously difficult to isolate and cultivate *in vitro* (Amann *et al*, 1995). The use of molecular biological techniques provides the opportunity to investigate microbial communities in landfill without cultivation (Embley & Widdick, 1991).

The microorganisms responsible for degradation in landfills can be divided into several trophic groups (Palmisano & Barlaz, 1996). One of these groups, the methanogens, is a diverse group of *Archaea* that are directly responsible for the production of methane. Methane is a major component of landfill gas. It is a 'greenhouse gas' and a potentially valuable energy source (Palmisano and Barlaz, 1996; Ritchie *et al*, 1997). However, little is known about the methanogens in landfill (Luton, 1996). The development and application of molecular biological techniques

to the investigation of the methanogenic *Archaea* in landfill was the main focus of this project.

1.2 Waste Management

A major concern for the UK and the rest of the world is how to manage the ever-increasing volumes of waste. The Department of the Environment, Transport and the Regions (DETR) estimate that about 423 million tonnes of waste are produced in the UK each year (Anon, 2000d). The reported total waste generation within the European Union (EU) and the European Free Trade Area increased by nearly 10% between 1990 and 1995 (Anon, 1999). This amounted to about 3.5 tonnes of solid waste per person in 1995 (Anon, 1999). Figure 1.1 shows the generation rates for MSW from 11 countries.

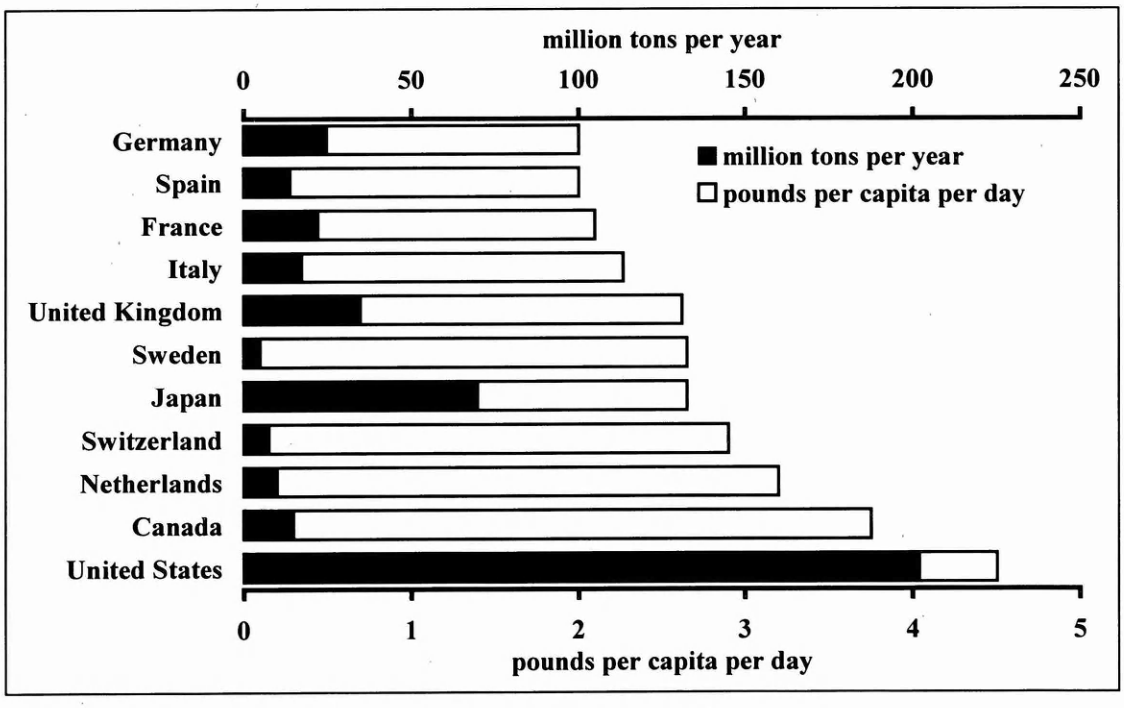


Figure 1.1 Generation of municipal solid waste by major countries
Reproduced from Anon (1997).

1.2.1 Sources of waste

Wastes are commonly categorised by their source. The major sources of solid waste are agriculture, mining and quarrying, construction and demolition, manufacturing, energy production, sludge from sewage treatment, dredging, waste from commercial premises and households (Anon, 1999; Read, 1999). Figure 1.2 shows the contribution of these sources to the total waste arisings in the United Kingdom.

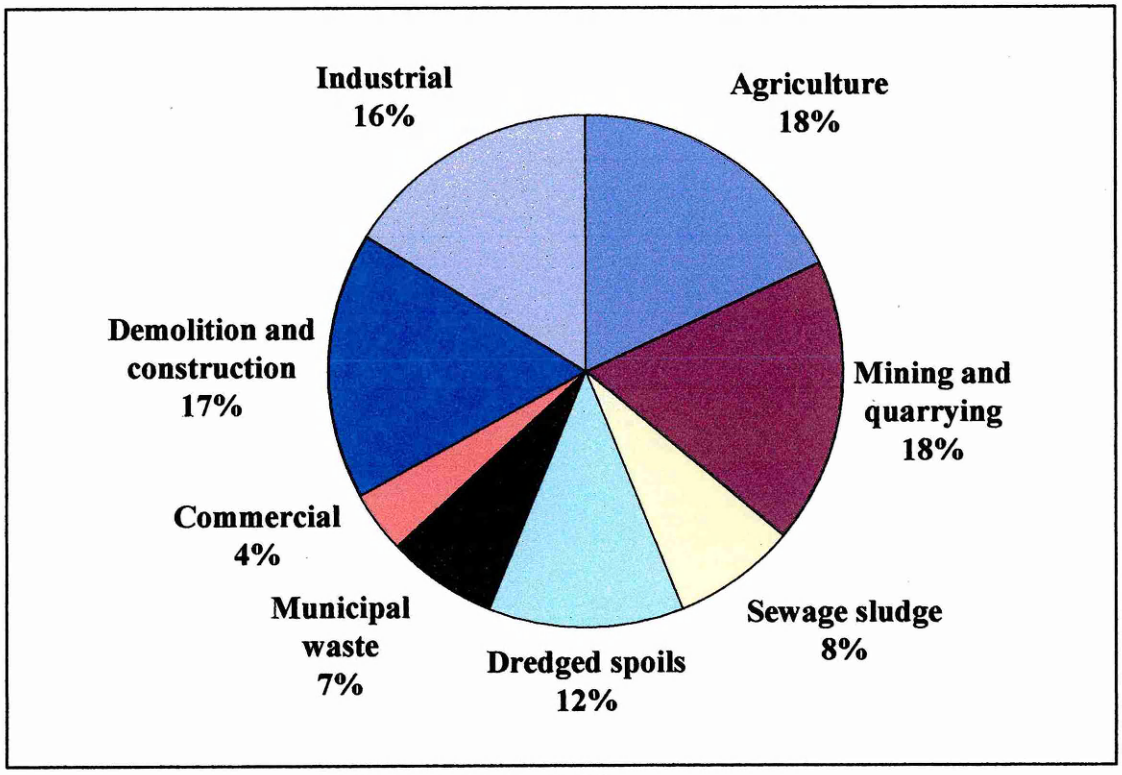


Figure 1.2 Estimated annual waste in the UK by sector
Reproduced from Anon (2000d).

1.2.2 The need for better waste management

The sheer quantity of waste represents an enormous loss of resources both in the form of materials and energy. Furthermore, management of this waste causes a variety of impacts on the environment, (Table 1.1).

Table 1.1 Potential environmental impacts of waste management options

	Air	Water	Soil	Landscape	Ecosystems	Urban areas
Landfill	Emissions of CH_4, CO_2; odours.	Leaching of salts, heavy metals, bio-degradable and persistent organics to groundwater	Accumulation of hazardous substances in soil	Soil occupancy; restrictions on other land uses	Contamination and accumulation of toxic substances in the food-chain	Exposure to hazardous substances
Composting	Emissions of CH_4, CO_2; odours			Soil occupancy; restrictions on other land uses	Contamination and accumulation of toxic substances in the food-chain	
Incineration	Emissions: SO_2, NO_χ, HCl, HF, NMVOC[a], CO, CO_2, N_2O, dioxins, dibenzofurans, heavy metals (Zn, Pb, Cu, As)	Deposition of hazardous substances on surface water	Landfilling of slags, fly ash and scrap	Visual intrusion; restrictions on other land uses	Contamination and accumulation of toxic substances in the food-chain	Exposure to hazardous substances
Recycling	Emissions of dust	Wastewater discharges	Landfilling of final residues	Visual intrusion		Noise
Transportation	Emissions of dust, NO_χ, SO_2; release of hazardous substances from accidental spills	Risk of surface water and groundwater contamination from accidental spills	Risk of soil contamination from accidental spills	Traffic	Risk of contamination from accidental spills	Risk of exposure to hazardous substances from accidental spills; traffic

[a] Non-methane volatile organic compounds

Reproduced from Anon (1995a).

Of the 106 million tonnes of waste produced by industry, commerce and households, most is still managed by disposal to landfill. Table 1.2 shows the percentage of waste going to different waste management options in 1998/99. We cannot continue to rely on landfill as we have done in the past. It has been estimated that 59% of current landfill capacity in the UK will be used by 2010 (Read, 1999). In certain parts of the UK, particularly South East England, it is becoming increasingly difficult to find sites for new landfills due to the scarcity of suitable land and public objections (Read *et al*, 1997). All of these factors point to the need to reduce the amount of waste generated and to divert waste away from landfill disposal towards alternative waste management options.

Table 1.2 Waste management in England and Wales 1998/99

	Landfill (%)	**Recovery (%) (recycling and composting)**
Industrial waste (excluding construction and demolition waste)	47	45 (39)
Commercial waste	66	33 (29)
Municipal waste	83	17 (9)

Source: (Anon, 2000e)

Figure 1.3 shows the trends in municipal waste management in England and Wales between 1996/97 and 1998/99. The most important change is the reduction in the proportion of municipal waste being landfilled from 85% to 82%. This decrease in landfilling is due mainly to increases in recycling, composting and incineration with energy recovery. By comparison, in 1998 the United States landfilled 55% of MSW, recovered 28% and incinerated 17% (Anon, 2000a).

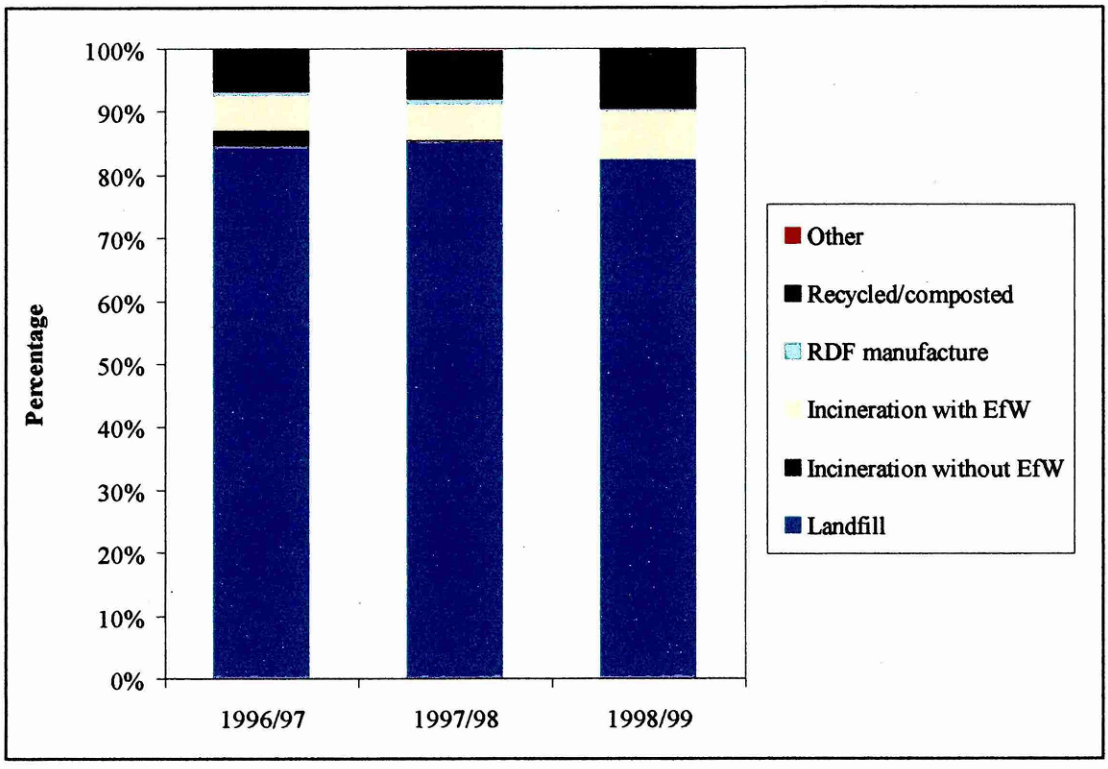


Figure 1.3 Municipal waste management in England and Wales: 1996/97, 1997/98 and 1998/99.
RDF = Refuse Derived Fuel, EfW = Energy from Waste.
Source: (Anon, 2000b; Anon, 2000c).

1.2.3 Options for waste management

At the heart of the UK Government's current waste management policies is the 'waste management hierarchy' (Anon, 2000e).

The waste management hierarchy

- Reduction
  - Re-use
    - Recovery
      - Disposal

The principle behind the waste management hierarchy is that the options at the top of the hierarchy should be considered before those further down the hierarchy. Disposal

options, of which landfill is the foremost example, should only be considered when options higher up the hierarchy have been exhausted.

Waste that cannot be prevented, reused or recovered still needs to be disposed of safely, minimising the environmental impact. The principal options for disposal are incineration and landfilling. Some value can still be recovered from incinerated or landfilled waste by utilising the heat from incineration or collecting landfill gas (Anon, 2000f). Landfill gas is formed from the decomposition of organic material in landfills and typically consists of 65% methane and 35% carbon dioxide. The gas can be used to fuel reciprocating engines or turbines to generate electricity, or used directly in kilns and boilers. Landfill operators are obliged to collect and treat landfill gas either by flaring or through energy recovery. At the end of 1998 there were 107 projects in the UK generating 200 megawatts of power from landfill gas (Anon, 2000f). Energy from waste can make an important contribution towards sustainable development as a source of renewable energy, reducing the use of fossil fuels and cutting emissions of greenhouse gases.

1.2.4 Incentives for better waste management

Waste legislation in the UK is driven by the need to manage waste safely and effectively and also by the UK's commitments to comply with international, particularly EU, legislation. One piece of legislation in particular will bring about major changes to waste management in the UK, the EU Landfill Directive (Council Directive 99/31/EC). The main requirements of the landfill directive are:

- targets for reduction of biodegradable municipal waste to landfill;

- banning co-disposal of hazardous and non-hazardous wastes, and requiring separate landfills for hazardous, non-hazardous and inert wastes;
- banning landfill of tyres (by 2003 for whole tyres, 2006 for shredded tyres)
- banning landfill of liquid wastes, infectious clinical waste and certain types of hazardous waste (e.g. explosive, highly flammable), all by 2001;
- provisions on the control, monitoring, reporting and closure of sites, which already form the backbone of waste management legislation in the UK (Anon, 2000e).

The targets for the reduction of biodegradable municipal waste going to landfill, which the UK is currently committed to achieving, are a reduction to 75% of 1995 levels by 2010, 50% by 2013 and 35% by 2020 (Anon, 2000e).

To achieve the requirements of the landfill directive and other European waste legislation the Government has put in place a number of instruments designed to reduce the amount of waste produced, and to increase re-use, recycling and energy recovery. These instruments include:

- The Waste and Resources Action Programme – This programme aims to identify and facilitate new uses for recycled materials, promote investment in reprocessing, provide a source of information for waste and recycling data, provide advice, guidance and technical support.
- Producer responsibility – This can be an effective tool for making producers (and others involved in the distribution and sale of goods) more aware of the environmental impact of the goods they produce and take more responsibility for those goods at the end of their lives. Voluntary agreements or mandatory obligations have been set up, or are being considered, in a number of sectors

including packaging, newspapers, junk mail, end-of-life vehicles, batteries and waste electrical and electronic goods.

- Landfill tax - In October 1996 a Landfill Tax was introduced in the UK. The stated purpose of the tax was 'to ensure that landfill waste disposal is properly priced so as to reflect its environmental cost' and 'to promote a more sustainable approach to waste management in which less waste is produced and more waste is either reused or has value recovered from it' (Morris *et al*, 1998).
- Landfill tax credit scheme – Landfill operators can claim up to 90% tax credit against donations they make to environmental bodies carrying out activities including: reclamation of polluted land, research and education activities to promote re-use and recycling, provision of public parks and amenities, and restoration of historic buildings.
- Landfill permits – The Government proposes to allocate tradable permits to local authorities, which will set tonnages of biodegradable municipal waste to be landfilled.
- Public awareness – Campaigns such as the 'are you doing your bit?' campaign raise public awareness of environmental issues including waste and recycling.

(Anon, 2000e).

1.3 Landfill

Landfill is the ultimate disposal option for waste that cannot be reduced, re-used, recycled, composted, incinerated or processed in some other manner. Landfills are needed for disposing of residues from recycling, composting, incineration and other processes. The increase in alternative waste management practices is diverting waste away from landfills. However, for the foreseeable future landfills will be a significant part of the waste management strategy of most countries.

1.3.1 Design and operation of a modern sanitary landfill

Modern sanitary landfills differ greatly from the open dumps of the past. The design and operating procedures of modern landfills have evolved over the last 20 years in response to increased awareness of their environmental impacts. The health, safety and aesthetic problems encountered at open dumps included rodents, flies, fires and odours. Sanitary landfills developed when controlled operation and disposal techniques such as daily cover and compaction were found to minimise many of these safety and aesthetic concerns (Reinhart & Townsend, 1998). Although measures such as daily cover, reduced the infiltration of water into the landfill, they were not sufficient to control the problems of leachate and gas production. Strict regulations now require modern landfills to have engineered systems in place, to prevent the uncontrolled release of leachate and gas from the landfill.

1.3.1.1 Design features of a modern landfill

The major components of a modern landfill are described below and illustrated in Figure 1.4.

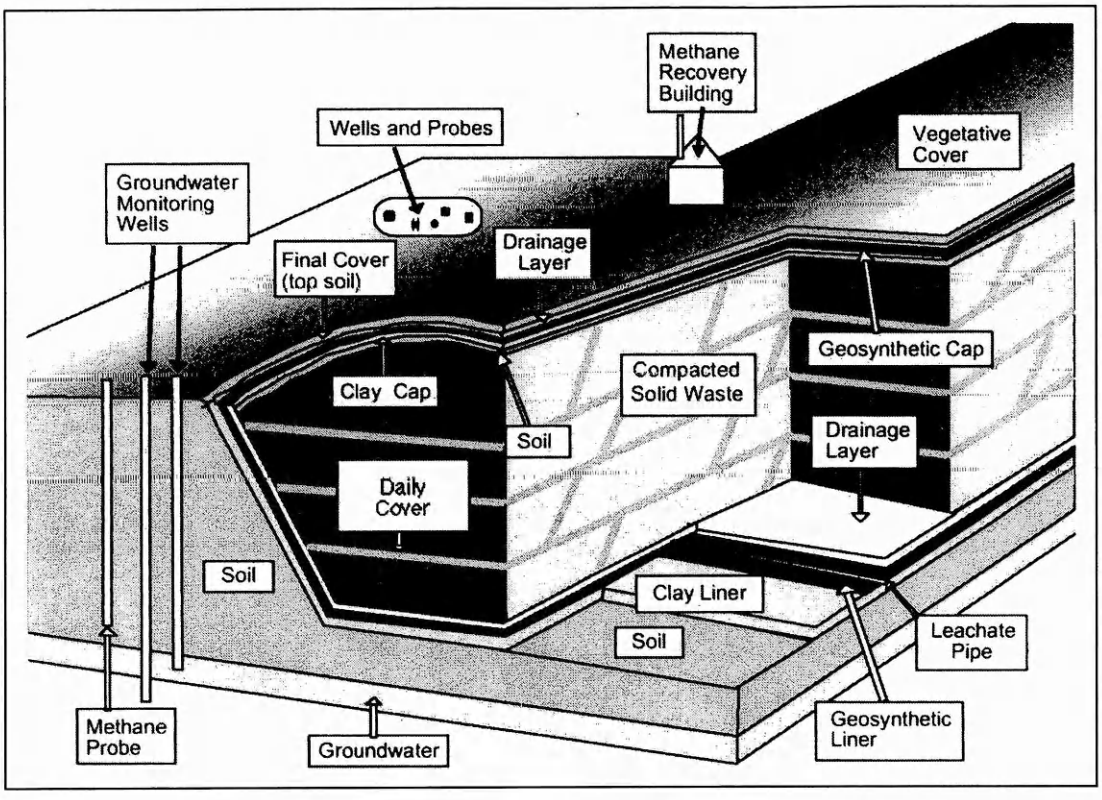


Figure 1.4 Schematic of a modern sanitary landfill.
Reproduced from O'Leary *et al* (1995).

- **Site:** Landfills are frequently sited in old quarries or other excavations. This has the benefits of keeping the waste out of sight and provides a cheap means of restoring the landscape once the landfill is covered over. Old parts of a quarry that are no longer being worked may be used for landfill, while minerals are still being extracted in another part of the site. Where an existing hole is not available, a site may be excavated to increase the available disposal volume per acre or waste may be deposited above the ground level, (landraising).
- **Liner:** The liner is a system of clay layers and/or geosynthetic membranes used to collect leachate and reduce or prevent contaminant flow to groundwater (O'Leary *et al*, 1995). Commonly the liner system consists of a layer of low permeability soil, typically a 1m thick layer of compacted clay. A geosynthetic membrane liner is often placed above the clay layer. A geomembrane is a thin sheet of plastic that

is highly impermeable to water and resistant to chemical attack. The most common type of geomembrane used in landfills is high-density polyethylene (HDPE) (Reinhart and Townsend, 1998). The soil and plastic together are referred to as a composite liner. The type of liner system appropriate for a landfill will depend on a number of factors, including, the geology and hydrogeology of the area, the proximity of drinking water sources, the types of waste deposited and the mode of operation of the landfill.

- **Leachate collection system:** Pipes are placed at the low areas of the liner to collect leachate for storage and eventual treatment and discharge. Leachate flow over the liner to the pipes is facilitated by placing a drainage blanket of soil or plastic netting over the liner. An alternative to collection pipes is a special configuration of geosynthetic materials that will hydraulically transmit leachate to collection points for removal (O'Leary *et al*, 1995). An additional layer of soil, baled waste or tyre chips may be placed above the leachate collection system to protect it from the equipment used to place waste in the landfill (Palmisano and Barlaz, 1996).
- **Daily cover:** At the end of each days operations the compacted waste is covered to minimise the attraction of rodents, wildlife, and disease-carrying insects; blowing of the refuse away from the landfill; infiltration of rain into the waste and contamination of stormwater runoff. The daily cover may be a thin, 15cm soil layer or plastic sheets that are rolled over the waste at the end of the day and removed prior to placement of additional waste (Palmisano and Barlaz, 1996).
- **Final cover:** Once a section of landfill is full a final cover is applied. The purpose of the final cover is to control infiltration of water, gas emission to the atmosphere, and erosion. In general the final cover consists of a layer of low

permeability soil, e.g. compacted clay, overlaid by a layer of soil that will support vegetation (Palmisano and Barlaz, 1996). Additional layers may be included, such as a drainage layer, and the surface of the landfill is contoured to prevent the build up of water on top of the landfill.

- **Gas control and recovery system:** A series of vertical wells or horizontal trenches containing permeable materials and perforated piping is placed in the landfill to collect gas for treatment or productive use as an energy source (O'Leary *et al*, 1995).
- **Gas monitoring probe system:** Probes are placed in the soil surrounding the landfill above the groundwater table to detect any gas migrating from the landfill (O'Leary *et al*, 1995).
- **Groundwater monitoring well system:** Wells are placed at appropriate locations and depth for taking water samples to verify that leachate is not escaping from the landfill in significant quantities (O'Leary *et al*, 1995; Palmisano and Barlaz, 1996).

1.3.1.2 Operation of a modern landfill

Modern landfills are classified into several categories depending on the types of waste deposited and the mode of operation. The new European Directive on landfilling of waste will require landfill sites to be categorised into one of three types depending on the type of waste received: hazardous, non-hazardous or inert (Anon, 2000f).

Landfills may also be categorised based on the operating principle. Operating principles include dilute and attenuate, containment, entombment, monofill, reusable

and bioreactor. The design of a landfill is dependent on its intended operating principle.

Landfills with only a rudimentary or no liner system rely on the dilute and attenuate principle. Leachate formed within the waste is allowed to migrate into the surrounding environment where it is attenuated in the surrounding geology, by biological and physico-chemical processes (Westlake, 1997). The leachate is diluted as it mixes with the groundwater. Older landfills and those accepting only inert or stable wastes may be operated in this way.

The underlying principle of the containment landfill is that leachate should not be allowed to migrate beyond the site boundary. In the developed world, containment landfill is now the accepted means of disposal to land, although the degree of engineering to achieve containment, and the management of water and other parameters varies considerably (Westlake, 1997). Landfill liner systems are never likely to provide absolute containment. However, the degree of containment provided by even a simple clay liner can be enough so that the environmental impact of leachate leakage from a landfill is at an acceptable level. In other circumstances, such as in a hazardous waste landfill, more effective containment would be required. The containment principle applies equally to gas control measures.

In landfills operated as entombment landfills, also known as dry-tomb or secure landfills, moisture is, as far as possible excluded from the waste, so that the waste will remain dry, will not decompose and will not produce leachate or gas (Westlake,

1997). An entombment landfill stores the deposited waste and prevents environmental impact for as long as the containment system remains intact.

Monofill landfills accept waste that cannot be processed by recycling, composting, energy recovery or incineration. These materials tend to be inert and may be assimilated more easily by the environment. Incinerator ash, and construction and demolition debris may be disposed of by monofill (Reinhart and Townsend, 1998).

Bioreactor landfills are operated in a manner that minimises environmental impact while optimising waste degradation processes. The fundamental process used for waste treatment in a bioreactor landfill is leachate recirculation. Recirculating the leachate through the waste creates an environment that is favourable for rapid microbial decomposition of the biodegradable waste (Reinhart and Townsend, 1998).

1.3.2 Waste composition

As stated in section 1.2, the wastes deposited in landfills come from a number of sources including households, businesses, the construction industry and other industries. The composition of these different waste streams varies greatly. The physical and chemical nature of the waste will affect its behaviour in the landfill, particularly its biodegradability. The main components of MSW in the UK are paper and cardboard, food and garden waste, plastics, glass and metals (Figure 1.5). It is the organic fraction of the waste that fuels the microbiological decomposition process. In MSW the organic fraction accounts for approximately 55% by dry weight of the waste, while the organic fraction of commercial and mixed industrial wastes are estimated to make up 66% and 62% by dry weight respectively (Anon, 1995b). The

principal biodegradable components of MSW are cellulose and hemicellulose. The other major organic component is lignin, which is recalcitrant under anaerobic conditions. As well as being recalcitrant, lignin can impede microbial access to the cellulose and hemicellulose, thereby reducing the overall degradability of the waste. Cellulose, hemicellulose and lignin account for 28-51%, 9-12% and 14-23% by dry weight of MSW respectively (Palmisano and Barlaz, 1996). Other biodegradable organic compounds present in smaller concentrations are protein, fats and soluble sugars.

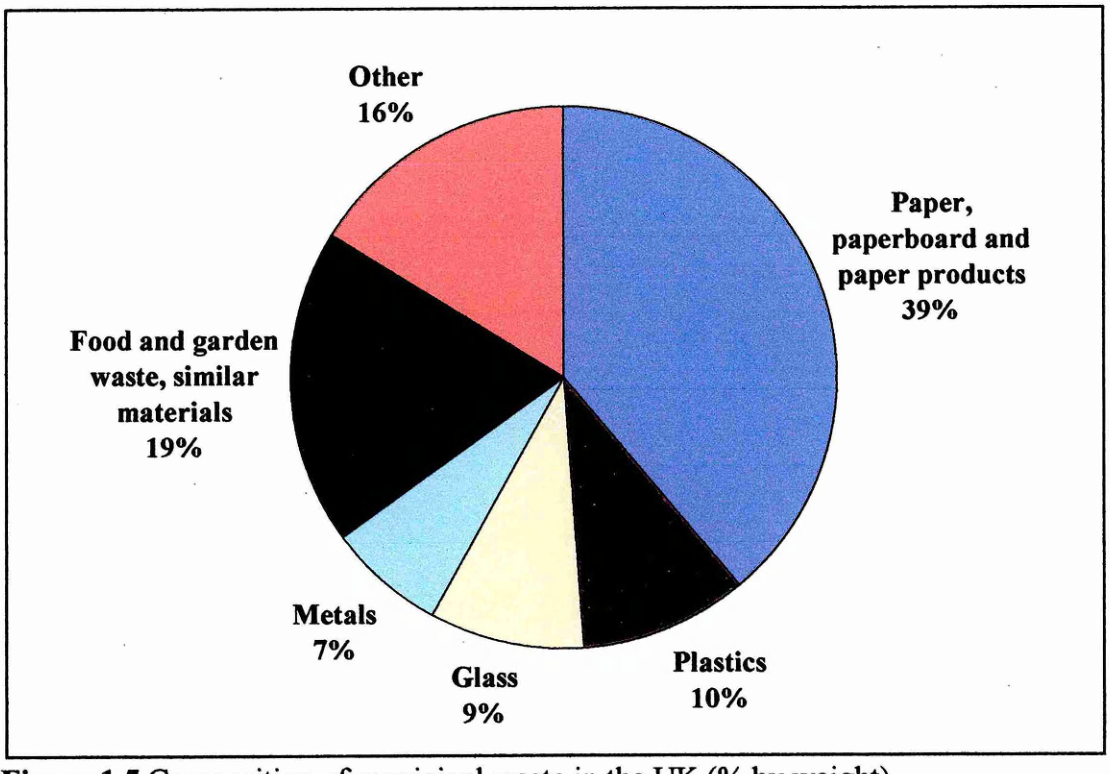


Figure 1.5 Composition of municipal waste in the UK (% by weight)
Source: (Anon, 1995a)

1.3.3 Biological decomposition in a landfill

The decomposition of the organic fraction of wastes within a landfill is a microbially mediated process that requires the co-ordinated activity of several trophic groups of microorganisms. The general pathway for decomposition in landfills is believed to be

the same as that occurring in other anaerobic ecosystems such as anaerobic digesters, rice paddies, marshes and the rumen. The process can be broken down into several stages each characterised by different groups of organisms, substrates and products, as illustrated in Figure 1.6.

1.3.3.1 The stages of decomposition

The first stage of the process is the hydrolysis of polymers (polysaccharides, proteins and lipids) to oligomers and monomers (sugars, amino acids, long-chain carboxylic acids and glycerol). This stage involves cellulolytic and other hydrolytic bacteria using the oxygen present in the waste. The duration of this aerobic phase depends on the availability of oxygen, which is influenced by management practices at the site, such as the degree of waste compaction, the depth of waste and the type of daily cover. As the oxygen becomes depleted other groups, i.e. facultative anaerobes and then obligate anaerobic microorganisms, supersede the aerobic microorganisms.

In the second stage, the complex polymers continue to be hydrolysed under anaerobic conditions and the hydrolysis products are fermented to short-chain carboxylic acids, succinate, lactate, etc. Some of these fermentation products, especially acetate, carbon dioxide, hydrogen and other one-carbon compounds, can be converted directly by methanogenic *Archaea* to methane and carbon dioxide (Schink, 1997). The third stage is characterised by the conversion of the other fermentation products (fatty acids longer than two carbon atoms, alcohols longer than one carbon atom, and branched-chain and aromatic fatty acids) to acetate, carbon dioxide, hydrogen and sometimes formate (Schink, 1997). Bacteria referred to variously as syntrophic, obligate proton-

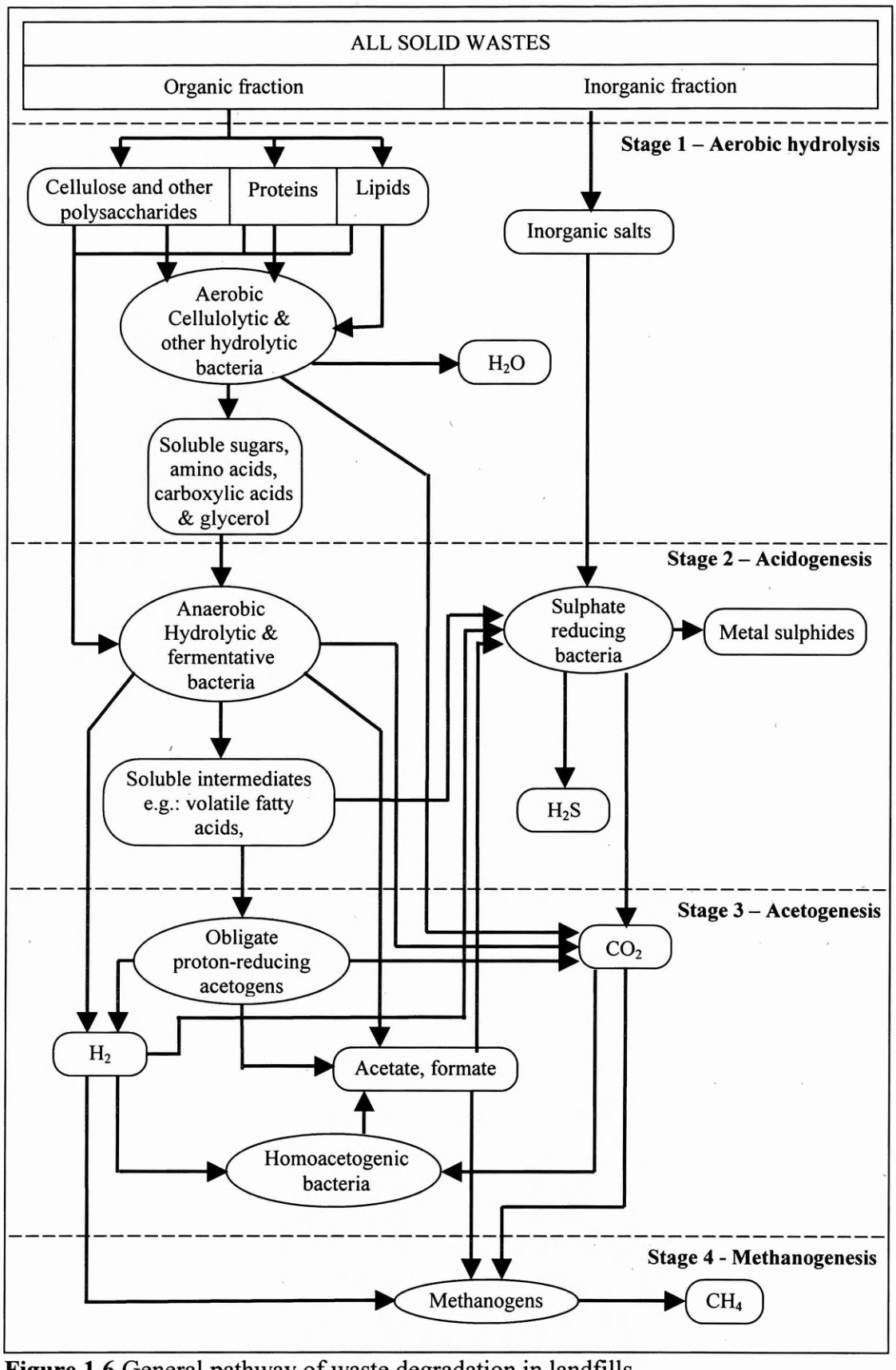


Figure 1.6 General pathway of waste degradation in landfills
Adapted from Anon (1995b) and Palmisano and Barlaz (1996).

reducing or H_2-producing acetogens carry out these reactions. Oxidation of fatty acids, alcohols, etc is only thermodynamically favourable at very low hydrogen concentrations. Thus, the obligate proton-reducing acetogenic bacteria only function in syntrophic association with hydrogen-utilising organisms such as methanogens or sulfate-reducing bacteria (Barlaz, 1997). Another group of acetogens, the homoacetogenic bacteria, convert carbon dioxide and hydrogen to acetate, though the significance of this reaction in the landfill ecosystem has not been established (Palmisano and Barlaz, 1996). They can also participate in sugar fermentation and degradation of special substrates such as *N*-methyl compounds or methoxylated phenols (Schink, 1994).

The fourth stage of the decomposition process is carried out by the methanogenic *Archaea*. Methanogens either convert acetate or carbon dioxide plus hydrogen to methane. A few other substrates such as formate and methanol may be utilised by some species of methanogen. It has been estimated that 60% of methane is generated from acetate in anaerobic environments (Ferry, 1992). However, the production of methane from acetate yields only 31kJ per mole CH_4 produced, whereas the conversion of H_2 plus CO_2 to CH_4 yields 135.6kJ per mole CH_4 produced (Palmisano and Barlaz, 1996). Thus, the growth of methanogens on acetate is relatively slow by comparison to growth on H_2 plus CO_2.

The route by which carbon and electrons flow from polymers such as cellulose to the final end products of anaerobic degradation such as methane and carbon dioxide is dependent on physical, chemical and biological factors. For example, if an abundant source of sulphate is available, such as the gypsum (calcium sulphate) in scrap

plasterboard, sulfate-reducing bacteria (SRB) can out-compete methanogens for hydrogen and produce hydrogen sulphide, metal sulphides and CO_2 as the end products of degradation (Palmisano and Barlaz, 1996). SRB are metabolically versatile, and a broad community of sulphate reducers can use all the products of primary fermentations and oxidise them to carbon dioxide and sulphide (Schink, 1997). As a consequence, complete oxidation of complex organic matter does not depend on syntrophic fermentations.

In a well balanced anoxic ecosystem in which an active hydrogen-utilising population maintains a low hydrogen partial pressure the primary fermenting bacteria will mainly produce acetate, carbon dioxide and hydrogen, rather than long-chain fatty acids or alcohols (Schink, 1994; Schink, 1997). Again, the reduced fermentation intermediates and the secondary-fermenting (syntrophic) bacteria will play only a minor role in the degradation process. However, an anoxic system may become unbalanced for a number of reasons. During the early stages of degradation the readily putrescible compounds such as proteins, lipids and starch are rapidly broken down to fatty acids, carbon dioxide and hydrogen by the hydrolytic and fermentative bacteria. The syntrophic and hydrogen-utilising groups may not have developed sufficiently by this stage leading to an accumulation of fatty acids, hydrogen and carbon dioxide. The high level of hydrogen inhibits the syntrophic bacteria leading to further accumulation of fatty acids, which in turn can cause a drop in pH, thus inhibiting the hydrogenotrophic methanogens even further (Anon, 1995b; Schink, 1997). Methanogens are most active in the pH range 6.8-7.4 (Palmisano and Barlaz, 1996). The consequence may be that methanogenesis ceases entirely and the

fermentation stops with the accumulation of huge amounts of ill-smelling fatty acids, a process known as acid-souring.

A low pH, which is inhibitory to methanogenesis, may also have the effect of increasing the role played by homoacetogens. Under standard conditions, methanogenic hydrogen oxidation yields more energy than homoacetogenic hydrogen oxidation. However, in sulphate-poor anoxic environments with slightly acidic pH homoacetogens may take over the function of hydrogenotrophic methanogens, provided that a low acetate concentration is maintained by aceticlastic methanogens (Schink, 1997). The ability of homoacetogens to compete against methanogens for hydrogen is also improved at low temperatures. At temperatures lower than 20°C, homoacetogens appear to take over significant parts of hydrogen oxidation in paddy soil and lake sediments (Fey & Conrad, 2000; Schink, 1997). The temperature in landfills is typically in the range 20-35°C (Anon, 1988).

Within the landfill site as a whole, all the stages of the degradation process may be occurring at the same time, at different rates in different parts of the landfill. This is the result of waste being deposited over a period of time (usually 5 – 10 years), and the heterogeneity of the waste, which results in microenvironments with different physical and chemical conditions and hence, rates of degradation.

1.3.3.2 Landfill gas and leachate generation

During all stages of decomposition a range of gases are generated. When the waste is buried carbon dioxide is generated by aerobic metabolism. Hydrogen is produced in addition to carbon dioxide as hydrolysis and fermentation continues. The methane

concentration in the gas gradually rises as acetogenesis and methanogenesis become established. When the methane content of the landfill gas is at its maximum the total gas generation also reaches its peak flow rate. At this point landfill gas typically consists of 40-70% methane with the balance being carbon dioxide plus trace amounts of gases such as hydrogen sulphide, water vapour, hydrogen and various volatile organic compounds (Reinhart and Townsend, 1998).

Leachate in landfills results from liquid migrating through the waste and extracting materials from the waste. Material is removed from the waste mass by leaching of inherently soluble material, leaching of soluble products of biological and chemical transformation, and washout of fines and colloids (Reinhart and Townsend, 1998). The characteristics of the leachate are highly variable, depending on the composition of the waste, rate of water infiltration, moisture content of the waste, and landfill design, operation and age. The polluting potential of leachate is due to the harmful and toxic compounds it may contain and other characteristics such as biological oxygen demand (BOD), chemical oxygen demand (COD), pH, etc, which can have a detrimental effect on surface and ground water. Harmful organic compounds detected in leachate include polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and organophosphates. Toxic metals such as cadmium, lead and nickel are also frequently found in landfill leachates. Like landfill gas composition, leachate characteristics such as BOD, COD, pH and the concentration of degradation intermediates such as volatile fatty acids (VFAs) can be indicative of the overall state of decomposition of the waste mass. As mentioned previously, landfills contain waste in various stages of decomposition and this will be reflected in gas and leachate

samples (Palmisano and Barlaz, 1996). Leachate migration also provides a mechanism for transport of microorganisms within a landfill.

1.3.3.3 Factors affecting decomposition in landfills

There are a number of physical, chemical and biological factors that can affect the decomposition process in landfilled waste. Physical factors include moisture content, moisture flow, particle size and temperature. Chemical factors include pH, type, concentration and availability of substrates, and the presence of recalcitrant or toxic compounds. The activity of the groups of microorganisms will also affect the environment in the landfill.

One of the most critical factors affecting the biodegradation of landfilled waste is moisture content. The moisture content of fresh refuse ranges from 15 to 45% and is typically about 20% on a wet weight basis (Palmisano and Barlaz, 1996). A number of studies have shown that the rate of decomposition in landfills increases with increasing moisture content. Suflita *et al* (1992) observed a correlation between moisture content and methanogenesis in samples from the Fresh Kills Landfill (the world's largest landfill). Rees & Viney (1982) also observed improved leachate quality, i.e. lower BOD, and increased gas production from water-saturated parts of a landfill, compared to drier areas.

Moisture flow and distribution also influence decomposition. As mentioned previously, water acts as a vector for nutrient and microbial transport in landfills. Water may exist in landfills as 'perched' water tables, stagnant zones, free flowing water or be virtually absent in 'dry zones' (Anon, 1988).

One way in which moisture content and hence decomposition in landfills may be influenced is through leachate recirculation. Indeed leachate recirculation has often been suggested as the means by which landfills could be operated as bioreactors with enhanced rates of degradation, methane production and stabilisation (Reinhart and Townsend, 1998). Studies with laboratory-scale landfill simulations and full-scale landfills have shown the effects of leachate recirculation. Komilis *et al* (1999) and Reinhart and Townsend (1998) have reviewed experiments conducted on leachate recirculation. ElFadel (1999) conducted experiments with cells, 30m by 30m and 15m deep, filled with municipal refuse. They demonstrated that leachate recirculation resulted in enhancement of gas generation and methane yields, and increased settlement rates.

A critical factor for methane production in landfills is pH. The optimal pH for methanogenesis is between 6.8 and 7.4, and only a slight drop in pH can result in a dramatic reduction in the rate of methane production (Anon, 1988). The effects of moisture content and pH on methanogenesis are closely linked. Moisture addition stimulates fermentative activity, which can lead to an accumulation of carboxylic acids and an acidic pH, which inhibits methanogenesis. Barlaz *et al* (1987) found that leachate neutralisation prior to recirculation enhanced methane production in laboratory-scale simulators.

Temperature affects decomposition in landfills by affecting microbial activity. The optimum growth temperature for most mesophilic methanogens is between 35 and 40°C (Sowers, 1995). However, the temperature in landfills is typically in the range

20°C to 35°C (Anon, 1988). During the aerobic stage of degradation temperatures inside the waste may rise as high as 70°C due to the heat produced from aerobic metabolism (Anon, 1988). Conversely, very little heat is released during anaerobic metabolism, around 86% of the energy in glucose is conserved in methane (Archer & Robertson, 1982). The low level of energy released by anaerobic metabolism means that landfill temperature declines once the aerobic phase has ended.

Another factor influencing microbial decomposition in landfills is bioavailability. For biodegradation to occur degradable waste components must be accessible to attack by microorganisms. As discussed earlier, the presence of molecules such as lignin, which is recalcitrant under anaerobic conditions, closely associated with degradable molecules such as cellulose, can limit the extent and rate of degradation by impeding the access of microorganisms and their hydrolytic enzymes to the cellulose. Similar situations can occur where other non-degradable materials such as plastic, glass and metal obstruct microbial access to degradable materials. Preferential degradation of a refuse component (e.g. by proteolysis) during the early stages of degradation, can result in nutrient loss as leachate, since no microbial population capable of utilising the solubilised material has developed by that stage (Anon, 1988). Leachate recirculation would return these nutrients to the landfill, increasing their potential bioavailability.

Decreased particle size would be expected to enhance degradation by exposing increased surface area to microorganisms. Particle size can be reduced through shredding before waste placement. Shredding promotes a more uniform waste, improves water distribution and settlement (Reinhart and Townsend, 1998). Various

studies have shown that shredding either increased the rate of decomposition or had no effect (Ham & Bookter, 1982).

The presence of certain organic compounds and heavy metals may have an inhibitory effect on waste decomposition. Sandaa *et al* (1999) observed decreases in the percentage of *Archaea* and differences in the community structure in samples of soil that had been amended with sludge containing high levels of cadmium, copper, nickel and zinc, compared to unamended soil samples.

The activity of the microbial groups involved in waste decomposition affects the landfill environment and influences the activity of other microbial groups. The activity of aerobic microorganisms removes oxygen creating a suitable environment for oxygen sensitive anaerobes, and raises the temperature in the landfill increasing microbial activity. Fermentative bacteria produce carboxylic acids, which lower the pH and in so doing inhibit methanogenesis. Methanogens and sulfate-reducing bacteria use hydrogen, thereby lowering the hydrogen concentration and making the metabolism of VFAs by proton reducing acetogens thermodynamically favourable. The activity of SRB may reduce methane generation by using hydrogen that otherwise would have been available to methanogens.

1.4 Microorganisms involved in decomposition of landfilled waste

As discussed earlier the microorganisms involved in waste degradation can be categorised by function. The principal functional groups of microorganisms are those involved in hydrolysis, fermentation, acetogenesis and methanogenesis. Other groups may also play a major role in degradation under specific conditions, e.g. SRB in the presence of sulphate.

1.4.1 Hydrolytic microorganisms

This group may be sub-divided into proteolytic, lipolytic and carbohydrate-metabolising activities. The decomposition of proteins and lipids in landfills is relatively insignificant compared to that of carbohydrates. Proteins and lipids account for an average of 3.4% and 5.7% of the organic content of solid waste by dry weight respectively, compared to carbohydrate (cellulose, hemicellulose, lignin, sugar and starch) which accounts for an average of 81.1% (ElFadel *et al*, 1997).

Cellulose is by far the most abundant carbohydrate in landfill. Municipal refuse typically contains 40-50% cellulose (Barlaz *et al*, 1989). Catabolism of cellulose in landfills is carried out by aerobic fungi, and both aerobic and anaerobic bacteria. In the upper layers of landfills and in freshly buried refuse, aerobic fungi and bacteria are responsible for the decomposition of cellulose. Pourcher *et al* (2001) identified the predominant groups of aerobic cellulolytic bacteria in landfill as belonging to the family *Bacillaceae*, and to the genera *Cellulomonas, Microbacterium* and *Lactobacillus*. Several species of anaerobic cellulolytic bacteria have been isolated from landfills. The first was *Cellulomonas fermentans*, isolated from samples taken at a depth of 23 metres from a municipal dumping ground in France (Bagnara *et al*,

1985). Westlake & Archer (1990) reported the isolation of six further species of anaerobic cellulolytic bacteria from landfill, belonging to the genera *Clostridium* and *Eubacterium*. All the isolates were found to produce a variety of end products from fermentation, including acetate, formate, pyruvate, ethanol, lactic acid, succinate, carbon dioxide and hydrogen. Some of these products, namely acetate, formate, CO_2 and H_2 may be used directly by methanogens, while the others would require conversion to other acids to be effective substrates for methanogenesis.

1.4.2 Fermentative or acidogenic bacteria

The microorganisms involved in fermentation may include the same organisms responsible for polymer hydrolysis, but also includes organisms that gain energy by the fermentation of compounds released by the activity of the hydrolytic organisms. Little information is available on the organisms and processes involved in the fermentative or acidogenic phase in landfill. It is assumed to be similar to other anaerobic environments such as the rumen and anaerobic digesters. In the rumen and anaerobic digesters, hydrolysis and fermentation of polymers such as cellulose, starch, proteins and lipids results in a range of products including soluble sugars, amino acids, triglycerides, long-chain fatty acids and carboxylic acids. These soluble intermediates are converted to VFAs by a variety of microorganisms.

Fermentative and hydrolytic bacteria have been enumerated in landfill by cultivation and measurement of specific enzyme activities. Jones & Grainger (1983) measured proteolytic, amylolytic, cellulolytic and lipolytic enzyme activities in samples of domestic refuse. They found high levels of protease and amylase activity during the early stages of degradation. Only low levels of cellulase activity were detected and

lipases were not detected. Palmisano *et al*, (1993) cultured 10^5-10^8 cfu(g dry wt)$^{-1}$ of fermentative bacteria from landfilled refuse. Only a small percentage (0-15%) was capable of producing extracellular hydrolytic enzymes for starch or protein degradation. Fermentative bacteria were identified in a laboratory-scale anaerobic reactor treating municipal landfill leachate (Frigon *et al*, 1997). The species identified included *Streptococcus gallinarum, Clostridium glycolicum, Clostridium bifermentans, Citrobacter amalonaticus, Bacteroides capillosus* and *Eubacterium* sp.

Molecular techniques have been used to investigate the diversity of bacteria in landfills. LloydJones & Lau (1998) used PCR, cloning, sequencing and phylogenetic analysis of 16S rDNA to investigate the microbial diversity in samples from a landfill heavily contaminated with PCBs and PAHs. None of the partial 16S rDNA sequences obtained were identical to known sequences obtained from cultivated bacterial species. Two clones showed 97.8% and 98.2% identity to 16S rDNA sequences of *Klebsiella planticola*. Species of the genus *Klebsiella* are facultatively anaerobic chemoorganotrophs, having both a respiratory and fermentative metabolism (Bergey & Holt, 1994). The majority, over 90% of the clones, could only be identified to sub-class or family level. In the low G+C Gram-positive taxon, sequences were observed that associated with the family *Clostridiaceae*, which are obligate anaerobes with fermentative metabolism (LloydJones and Lau, 1998). 16S rDNA sequences were also detected that were most similar to species of the anaerobic fermentative genus *Acetivibrio*, and to an anaerobic, hydrocarbon-degrading strain of *Bacillus benzoevorans*.

1.4.3 Acetogenic bacteria

In anaerobic environments, a wide range of bacteria produce acetate as an end product of fermentation. However, two groups of bacteria have been defined that produce acetate as the sole or predominant end product from fermentative metabolism. The syntrophic or obligate-proton reducing acetogens are involved in the conversion of VFAs, alcohols, etc to acetate, hydrogen and carbon dioxide, while the homoacetogenic bacteria utilise a wide range of substrates including H_2 and CO_2 to produce acetate.

1.4.3.1 Syntrophic fatty acid-oxidising acetogens

Very few studies have investigated the syntrophic acetogenic bacteria in landfills. It has been assumed that the species in landfill are similar to those found in other anaerobic systems (Anon, 1988; Archer and Robertson, 1982). A study of the microbiology and chemistry of six landfill test cells detected between 2.0×10^2 and 3.5×10^4 acetogens g^{-1} of landfill material in samples taken from a range of depths within the test cells (Anon, 1992). Acetogens were only detected in 9 out of 56 samples analysed and in three out of the six test cells. The fatty acids propionate, iso-butyrate, butyrate, iso-valerate and valerate were detected in all samples in concentrations ranging from 0 to 260mM. Butyrate showed the highest concentration in all the test cells. Acetate was also detected in concentrations from 1 to 20mM. Lay *et al* (1998b) enumerated methanogens and acetogens in sludge taken from laboratory-scale simulators of a landfill bioreactor treating the organic fraction of municipal solid wastes. The acetogen population in the landfill simulators increased by five to six orders of magnitude over a period of 300 days.

Seven genera of syntrophic, fatty acid-oxidising, acetogenic bacteria have been described, *Syntrophobacter, Syntrophomonas, Syntrophus, Syntrophospora, Thermosyntropha, Smithella* and *Syntrophothermus*. These genera are from two taxonomic groups, the delta sub-class of the Proteobacteria and the low G+C sub-class of Gram positive bacteria (Table 1.3). The delta Proteobacteria includes the majority of sulfate-reducing bacteria, while the low G+C Gram positive bacteria includes the clostridia, species of *Eubacterium* and the sulfate-reducing genus *Desulfotomaculum*. The majority of described species of syntrophic acetogens have been isolated from anaerobic wastewater digesters, operated at both mesophilic and thermophilic temperatures. The exceptions are *Syntrophospora bryantii* isolated from marine sediment and *Thermosyntropha lipolytica* isolated from alkaline hot springs of Lake Bogoria, Kenya (Stieb & Schink, 1986; Svetlitshnyi *et al*, 1996). As a group, the syntrophic acetogens typically: grow optimally between 30 and 37°C, neutral pH, produce acetate, hydrogen and carbon dioxide as the principal products of fermentation, and utilise straight-chain fatty acids plus a few branched or aromatic fatty acids as substrates in coculture with a hydrogen-utilising partner.

A number of syntrophic bacteria have also been described that catalyse syntrophic substrate oxidation of compounds other than fatty acids, via interspecies hydrogen transfer. These include: *Desulfovibrio vulgaris, Thermoanaerobium brockii* and species of *Pelobacter*, all of which oxidise ethanol; the glycolate oxidising *Syntrophobotulus glycolicus*; *Syntrophococcus succromutans*, a carbohydrate oxidiser; and the acetate oxidising species, *Clostridium ultunense* and

Table 1.3 Characteristics of syntrophic acetogenic bacteria.

Species	Taxonomic group	Substrates used		Principal products	Optimum growth conditions		Isolation source
		Coculture	Alone		pH	Temp	
Syntrophobacter wolinii	Delta Proteobacteria	Pr	Pr+SO_4	-	-	35	Anaerobic sewage digester
Syntrophobacter pfennigii	Delta Proteobacteria	Pr, La, Po	(La or Pr)+SO_4	Ac, CO_2	7.0-7.3	37	Municipal sewage sludge, anaerobic
Syntrophobacter fumaroxidans	Delta Proteobacteria	Pr	Pr, H, F, Sc with SO_4 or Fu, Fu, Ma, As, Py	Ac, CO_2, Sc	7	37	Sugar refinery wastewater digester
Syntrophus buswellii	Delta Proteobacteria	Bz	Cr	Ac, Bu	-	37	Municipal anaerobic digester
Syntrophus gentianae	Delta Proteobacteria	Bz	Hy, Ge	-	-	-	-
Syntrophus aciditrophicus	Delta Proteobacteria	Bz, C_4-C_{18}, UFA	Cr	Ac, H, F	-	35	Municipal anaerobic sewage digester
Smithella propionica	Delta Proteobacteria	Pr, Bu, Cr, Ma, Fu	Cr	Ac, H, Bu	6.8	34	Domestic sewage sludge
Syntrophomonas wolfei subsp. Wolfei	Low G+C Gram-positive bacteria	C_4-C_8, iC_7	C_4-C_6, UFA, Cr	-	-	30-37	Anaerobic digester sludge
Syntrophomonas wolfei subsp. saponavida	Low G+C Gram-positive bacteria	C_4-C_{18}	Cr	Ac, Pr, H_2S, H	-	30-37	Anaerobic sewage digester
Syntrophomonas sapovorans	Low G+C Gram-positive bacteria	C_4-C_{18}, O, E, L	-	-	7.3	35	Mesophilic sewage digester
Syntrophospora bryantii	Low G+C Gram-positive bacteria	C_4-C_{11}, 2MB	Cr	-	6.5-7.5	28-34	Marine sediment
Thermosyntropha lipolytica	Low G+C Gram-positive bacteria	C_4-C_{18}, O, L, Ta	YE, Tr, Ca, Cr, Be, Py, Ri, Xy	Ac, H, F, Pr	8.1-8.9	60-66	Alkaline hot spring
Syntrophothermus lipocalidus	Low G+C Gram-positive bacteria	C_4-C_{10}, iC_4	Cr	H, Ac, Pr	6.5-7.0	55	Thermophilic digested anaerobic sludge

Abbreviations: Ac, acetate; As, aspartate; Be, betaine; Bu, butyrate; Bz, benzoate; C, carbon number of the fatty acid; Ca, casamino acids; CO_2, carbon dioxide; Cr, crotonate; E, elaidate; F, formate; Fu, fumarate; Ge, gentisic acid; H, hydrogen; H_2S, hydrogen sulfide; Hy, hydroquinone; iC, iso-branched fatty acid; La, lactate; L, linoleate; Ma, malate; 2MB, 2-methylbutyrate; O, oleate; Po, propanol; Pr, propionate; Py, pyruvate; Ri, ribose; Sc, succinate; SO_4, sulphate; Ta, triacylglycerides; Tr, typtone; UFA, unsaturated fatty acid; Xy, xylose; YE, yeast extract.
References: (Boone & Bryant, 1980; Harmsen *et al*, 1998; Harmsen *et al*, 1993; Jackson *et al*, 1999; Liu *et al*, 1999; Lorowitz *et al*, 1989; McInerney, 1992; Sekiguchi *et al*, 2000; Sekiguchi *et al*, 1998; Svetlitshnyi *et al*, 1996; Wallrabenstein *et al*, 1995a; Wallrabenstein *et al*, 1994; Wallrabenstein *et al*, 1995b; Zhao *et al*, 1990).

Thermacetogenium phaeum (Hattori *et al*, 2000; Krumholz & Bryant, 1986; Schink, 1997; Schnurer *et al*, 1996). The acetate oxidisers can also operate as homoacetogens, generating acetate from CO_2 and H_2, depending on the concentrations of acetate and hydrogen (Hattori *et al*, 2000; Schnurer *et al*, 1996).

1.4.3.2 Homoacetogenic bacteria

Homoacetogenic bacteria as a group are characterised by their ability to catalyse the reduction of two CO_2 molecules to acetate as their typical fermentation product (Diekert, 1992; Schink, 1994). Also, by their use of the acetyl-CoA pathway as their predominant: 1) mechanism for the reductive synthesis of acetyl-CoA from CO_2; 2) terminal electron-accepting, energy-conserving process; and 3) mechanism for the synthesis of cell carbon from CO_2 (Drake, 1994). They are all strictly anaerobic members of the domain *Bacteria*. In most other respects they are a very diverse group.

The homoacetogenic bacteria includes, Gram-positive and Gram-negative types, endospore formers, rod-shaped and coccoid forms, motile and non-motile organisms, and those that are psychrophilic, mesophilic and thermophilic (Diekert, 1992). The different species of homoacetogens are often only distantly related. The classification of homoacetogens is made more difficult by the fact that some species are classed in genera that include non-acetogens (Diekert, 1992). For example, several clostridia are homoacetogenic. Currently, there are around 17 different genera containing homoacetogenic species (Table 1.4).

Table 1.4 Genera of homoacetogenic bacteria

Genera	No. of species	T_{opt} (°C)	Isolation source
Acetitomaculum	1	38	Rumen fluid, steer
Acetoanaerobium	2	37	Sediment and oil field
Acetobacterium	7	27-30	Various
Acetohalobium	1	38-40	Saline lagoon
Acetomicrobium	2	55, 70	Sewage sludge
Acetonema	1	30	Wood-eating termite gut
Clostridium	7	30	Various
Eubacterium	1	30-37	Rumen fluid, sheep
Finegoldia	1	37	-
Micromonas	1		-
Moorella	3	55-60	Horse manure and hot springs
Oxobacter	1	37	Rumen, cattle
Ruminococcus	2	35-37	Human septicemia and human faeces
Sporomusa	7	30	Various
Syntrophococcus	1	35-42	Rumen fluid, steer
Thermacetogenium	1	58	Anaerobic thermophilic wastewater
Thermoanaerobacter	1	60-65	Lake sediment

References: (Bernalier *et al*, 1996; Drake, 1994; DSMZ, 2001; Hattori *et al*, 2000; Schink, 1994)

Homoacetogenic bacteria are probably the most versatile of the strictly anaerobic microorganisms. Most of them are able to grow on a variety of different substrates, including sugars, some one carbon compounds and alcohols, many of which are unfavourable energy sources under anaerobic conditions (Diekert, 1992). They can carry out incomplete oxidations of reduced fermentation products released by other fermenting bacteria (Schink, 1994). In addition to forming acetate from CO_2 and H_2, which has been reported for nearly all homoacetogens, some strains have been shown to carry out the reverse reaction, cleaving acetate to form H_2 and CO_2 in syntrophic association with a hydrogen-utilising species. *Clostridium ultunense*, isolated from swine manure, and *Thermacetogenium phaeum*, isolated from an anaerobic reactor treating wastewater from a kraft-pulp production plant, both oxidised acetate when in coculture with a hydrogenotrophic methanogen (Hattori *et al*, 2000; Schnurer *et al*,

1996). These two species also grew homoacetogenically with a range of carbon sources.

Homoacetogenic bacteria have been isolated from a wide range of anoxic environments (Table 1.4). A number have been isolated from sewage sludge, sewage digesters and waste water from industrial processes, but there are no reports of homoacetogens identified in landfill. The importance of homoacetogens in anaerobic ecosystems is unclear. They connect the pool of one-carbon compounds and hydrogen to that of acetate, both of which are the key substrates of the two main physiological groups of methanogens. Due to their metabolic versatility, they can also participate in sugar fermentation and degradation of special substrates such as *N*-methyl compounds or methoxylated phenols (Schink, 1997). In certain environments that are not strictly anoxic, such as the hind-gut of termites and periodically flooded soils and rice paddies, homoacetogens can out compete the energetically more favoured methanogens for hydrogen. This may be because methanogens, in general, are more oxygen-sensitive than homoacetogens (Schink, 1994). Under standard conditions, methanogenic hydrogen oxidation yields more energy than homoacetogenic hydrogen oxidation. However, in situations with slightly acidic pH and/or temperatures lower than 20°C, homoacetogens are able to compete successfully with methanogens for hydrogen (Schink, 1997). The success of homoacetogens in natural anoxic environments appears to be due their metabolic versatility, i.e. their ability to switch between various substrates or to use them simultaneously (Schink, 1994).

1.4.4 Sulfate-reducing bacteria

The class of microorganisms, which conduct dissimilatory sulphate reduction, that is the process in which sulphate acts as an oxidising agent for the dissimilation of organic matter, are commonly referred to as the sulfate-reducing bacteria (SRB) (Postgate, 1984). They are a physiologically diverse group, able to use a wide variety of electron donors, including hydrogen, formate, acetate, fatty acids, ethanol and benzoate (Devereux *et al*, 1989). Some genera, e.g. *Desulfovibrio* and *Desulfotomaculum*, are able to use a variety of simple carbon compounds, but are only capable of incomplete oxidation of these substrates, e.g. lactate to acetate. Other genera are nutritionally very limited, but are capable of complete oxidation, e.g. *Desulfobacter* is specific for acetate, which it oxidises to carbon dioxide (Voordouw & Wall, 1992). Due to their metabolic versatility, SRB are able to compete with methanogens, acetogens and fermenters for some substrates, but not with hydrolytic species, since carbohydrates in monomeric or polymeric form cannot in general be used by SRB (Voordouw and Wall, 1992).

There are greater than 28 genera of SRB . All except two of these genera are Gram negative, belonging to the delta subclass of the Proteobacteria. The One Gram positive genus is *Desulfotomaculum*. There are three genera of thermophilic sulfate-reducing *Bacteria* and one genera of thermophilic sulfate-reducing *Archaea*, *Archaeoglobus* (DSMZ, 2001; Voordouw and Wall, 1992). SRB are ubiquitous in the environment. They have been isolated from environments as diverse as oil field water and the human oral cavity (Langendijk *et al*, 1999; Tardy-Jacquenod *et al*, 1996). The activity of SRB in landfill was observed indirectly by the inhibition of methane production from samples of landfill containing high concentrations of sulphate (Kim

et al, 1997; Suflita *et al*, 1992). Daly *et al* (2000) used PCR and oligonuleotide probes for the 16S rRNA gene to detect six phylogenetic subgroups, covering nine genera of SRB, in samples of leachate from seven municipal landfills in England. The group consisting of the genus *Desulfobacterium* was the only group not detected in any of the leachate samples.

1.4.5 Methanogens

The methanogenic *Archaea* are a large and diverse group that is united by three features: 1) they form large quantities of methane as the major product of their energy metabolism; 2) they are strict anaerobes; 3) they are members of the domain *Archaea* and only distantly related to the *Bacteria* (Whitman *et al*, 1992). Table 1.5 shows some characteristics of 93 described species of methanogens.

1.4.5.1 Metabolism of methanogens

The energy metabolism of all methanogens involves the conversion of a limited number of substrates to methane. The major substrates are H_2 + CO_2, formate and acetate. In addition, some species use other simple carbon compounds such as methanol, methylamines and some alcohols as substrates (Table 1.5). All of these substrates are converted stoichiometrically to methane. This is different from the so-called 'minimethane' producers, anaerobic bacteria that produce very small amounts of methane from side reactions of their normal metabolism (Whitman *et al*, 1992).

The methanogenic *Archaea* can be divided into three groups by the types of compounds they are able to utilise as substrates. The first group, the hydrogenotrophs, obtain energy by the reduction of CO_2, with hydrogen, formate or

Table 1.5 Characteristics of the methanogenic *Archaea*

Organism	Substrates	Optimum growth conditions			Isolation source
		pH	temp (°C)	NaCl (M)	
Order *Methanobacteriales*					
Fam. *Methanobacteriaceae*[T]					
Methanobacterium[T]					
M. alcaliphilum	H	8.4	37	0.012	alkaline lake sediment
M. bryantii	H, 2P, 2B	6.9-7.2	37-39	0.26	sewage digester
M. defluvii	H, F	6.5-7.0	60-65	nd	methacrylic waste digester
M. espanolae	H	5.6-6.2	35	nd	kraft mill sludge
M. formicicum[T]	H, F, 2P, 2B	6.6-7.8	37-45	0.25	sewage digester
M. ivanovii	H	7.0-7.4	45	0.19	sewage digester
M. oryzae	H, F	7.0	40	0.09	rice field soil
M. palustre	H, F, 2P, CP	7.0	37	0.2	peat bog
M. subterraneum	H, F	7.8-8.8	20-40	0.2-1.25	deep granitic groundwater
M. thermoaggregans	H	7.0-7.5	65	0	cattle pasture
M. thermoflexum	H, F	7.9-8.2	55	nd	methacrylic waste digester
M. thermophilum	H	8.0-8.2	62	nd	digester methane tank
M. uliginosum	H	6.0-8.5	40	nd	marsh sediment
Methanobrevibacter					
M. arboriphilicus	H, F	7.8-8.0	30-37	nd	cotton wood tree
M. curvatus	H	nd	nd	nd	termite hindgut
M. cuticularis	H	nd	nd	nd	termite hindgut
M. filiformis	H	7.0-7.2	30	nd	termite hindgut
M. oralis	H	6.9-7.4	36-38	nd	human oral cavity
M. ruminantium[T]	H, F	6.3-6.8	37-39	nd	bovine rumen
M. smithii	H, F	6.9-7.4	37-39	nd	sewage digester
Methanosphaera					
M. cuniculi	H/Me	6.8	35-40	nd	rabbit rectum
M. stadtmanae[T]	H/Me	6.5-6.9	36-40	nd	human faeces
Methanothermobacter					
M. marburgensis	H, (F)	6.8-7.4	65	0.5	anaerobic sewage digester
M. thermoautotrophicus[T]	H	7.2-7.6	65-70	0.6	sewage digester
M. wolfei	H, F	7.0-7.7	55-65	<1.7	sewage/river sediment
Fam. *Methanothermaceae*					
Methanothermus[T]					
M. fervidus[T]	H	6.5	83	nd	solfataric hot spring
M. sociabilis	H	6.5	88	nd	solfataric mud
Order *Methanococcales*					
Fam. *Methanococcaceae*[T]					
Methanococcus[T]					
M. maripaludis	H, F	6.8-7.2	35-39	0.2-0.6	marine marsh sediment
M. vannielii[T]	H, F	7.0-9.0	36-40	0.1	marine sediment
M. voltae	H, F	6.7-7.4	32-40	0.2-0.6	estuarine sediment
M. thermolithotrophicus	H, F	6.5-7.5	65	0.3-0.7	thermal coastal sediment
M. fervens	H	6.5	85	0.52	deep-sea hydrothermal vent
M. infernus	H	6.5	85	0.43	deep-sea hydrothermal vent
M. jannaschii	H	6.0	85	0.3-0.7	marine hydrothermal vent
M. vulcanius	H	6.5	80	0.43	deep-sea hydrothermal vent
M. igneus	H	5.7	88	nd	marine hydrothermal vent

Table 1.5 (continued)

Organism	Substrates	Optimum growth conditions			Isolation source
		pH	temp (°C)	NaCl (M)	
Order *Methanomicrobiales*					
Fam. *Methanomicrobiaceae*[T]					
Methanomicrobium[T]					
M. mobile	H, F	6.1-6.9	40	nd	bovine rumen
Methanoculleus					
M. bourgensis[T]	H, F	7.4	37	<0.18	tannery waste digester
M. marisnigri	H, F, 2P, 2B	6.6	20-25	0.1	marine sediment
M. oldenburgensis	H, F	7.5-8.0	45	0.043-0.17	river sediment
M. olentangyi	H	7.0	37	0.2	river sediment
M. palmolei	H, F, 2P, 2B, CP	6.9-7.5	40	nd	palm-oil plant wastewater
M. thermophilus	H, F	7.0	55	0-0.3	thermal marine sediment
Methanolacina					
M. paynteri[T]	H, F, 2P, 2B, CP	6.6-7.2	40	0.15	marine sediment
Methanogenium					
M. cariaci[T]	H, F	6.8-7.3	20-25	0.5	marine sediment
M. frigidum	H, F	6.5-7.9[b]	15	0.35-0.6	Antarctic lake
M. frittonii	H, F	7.0-7.5	57	0	lake sediment
M. organophilum	H, F, E, 1P, 2P, 2B	6.4-7.3	30-35	0.3	marine sediment
Methanofollis					
M. liminatans	H, F, 2P, 2B	7.0	40	0	industrial wastewater
M. tationis[T]	H, F	7.0	37-40	<0.3	solfataric hot pool
Fam. *Methanospirillaceae*					
Methanospirillum					
M. hungatei[T]	H, F	6.6-7.4	30-37	nd	sewage sludge
Fam. *Methanocorpusculaceae*					
Methanocorpusculum[T]					
M. aggregans	H, F	6.4-7.2	35-37	<0.18	sewage digester
M. bavaricum	H, F, 2P, 2B, CP	7.0	37	0	sugar plant wastewater
M. labreanum	H, F	7.0	37	0-0.2	tar pit lake
M. parvum[T]	H, F, 2P, 2B	6.8-7.5	37	0-0.8	whey digester
M. sinense	H, F	7.0	30	0	distillery wastewater
Methancalculus					
M. halotolerans[T]	H, F	7.6	38	0.86	oil well
M. pumilus	H, F	6.5-7.5	35	0.17	Japanese sea-based landfill
Fam. *Methanoplanaceae*					
Methanoplanus					
M. endosymbiosus	H, F	6.6-7.1	32	0.25	marine ciliate
M. limicola[T]	H, F	7.0	40	0.1-1.0	drilling swamp
M. petrolearius	H, F, 2P	7.0	37	0.17-0.52	African oil well

Table 1.5 (continued)

Organism	Substrates	Optimum growth conditions			Isolation source
		pH	temp (°C)	NaCl (M)	
Order *Methanosarcinales*					
Fam. *Methanosarcinaceae*					
Methanosarcina[T]					
M. acetivorans	AC, ME, MA	6.5-7.5	35-40	0.2	marine sediment
M. barkeri[T]	H, AC, ME, MA, PY	6.5-7.5	30-40	nd	sewage digester
M. mazei	AC, ME, MA	6.5-7.5	30-40	0.3-0.7	sewage digester
M. semesiae	DS, ME, MA, MT	6.5-7.5	30-35	0.2-0.6	mangrove sediment
M. siciliae	ME, MA, DS	6.5-6.8	37	0.4-0.6	lake sediment
M. thermophila	AC, ME, MA	6.0	45-50	nd	sewage digester
M. vacuolata	H, AC, ME, MA	7.5	40	nd	methane tank sludge
Methanolobus					
M. bombayensis	ME, MA, DS				sea sediment
M. taylorii	ME, MA, DS	8.0	37	nd	estuarine sediment
M. tindarius[T]	ME, MA	6.5	37	0.5	lake sediment
M. vulcani	ME, MA	7.0	37	nd	submarine fumarole
Methanococcoides					
M. burtonii	ME, MA	nd	23.4	nd	Antarctic lake water
M. methylutens[T]	ME, MA	7.0	30-35	0.2-0.6	marine sediment
Methanohalophilus					
M. halophilus	ME, MA	7.4	26-36	1.0-1.7	marine cyanobacterial mat
M. mahii[T]	ME, MA	7.4	35-37	1.0-2.5	saline lake sediment
M. oregonense	ME, MA, DS	8.6	35	nd	saline alkaline aquifer
M. portucalensis	ME, MA	6.5-7.5	40	nd	solar salt pond
M. zhilinae	ME, MA	9.2	45	0.5-1.0	alkaline lake sediment
Methanohalobium					
M. evestigatum[T]	ME, MA	7.0-7.5	50	4.3	salt lagoon sediment
Methanomicrococcus					
M. blatticola	ME, MA	7.2-7.7	39	<0.1	cockroach hindgut
Methanomethylovorans					
M. hollandica	ME, MA, MT, DS	6.5-7.0	34-37	0-0.04	freshwater pond sediment
Fam. *Methanosaetaceae*					
Methanosaeta					
M. concilii[T]	AC	7.1-7.5	35-40	nd	pear waste digester
Methanothrix					
M. thermoacetophila	AC	6.0-7.0	60-65	nd	thermal lake mud
M. thermophila	AC	7.4-7.8	35-40	nd	thermophilic sludge digester
Order *Methanopyrales*					
Fam. *Methanopyraceae*					
Methanopyrus					
M. kandleri[T]	H	6.5	98	0.25	geothermal marine sediment

Key: H = hydrogen+carbon dioxide; F = formate; AC = acetate; ME = methanol; MA = methylamines; H/ME = methanol reduction with hydrogen; E = ethanol; 1P = 1-propanol; 2P = 2-propanol; 2B = 2-butanol; CP = cyclopentanol; PY = pyruvate; DS = dimethylsulfide; MT = methanethiol.
T = type family of the order; type genus of the family; type species of the genus.
nd = not determined.
[b] Only a range reported.
References: (Anon, 1988; Blotevogel *et al*, 1991; Blotevogel & Fischer, 1985; Boone *et al*, 1993; DSMZ, 2001; Ferrari *et al*, 1994; Franzmann *et al*, 1997; Franzmann *et al*, 1992; Garcia, 1990; Garcia *et al*, 2000; Jeanthon *et al*, 1999b; Jeanthon *et al*, 1998; Jones *et al*, 1987; Joulian *et al*, 2000; Kadam *et al*, 1994; Kotelnikova *et al*, 1998; Leadbetter & Breznak, 1996; Leadbetter *et al*, 1998; Lomans *et al*, 1999; Lyimo *et al*, 2000; Mori *et al*, 2000; Ni & Boone, 1991; Ollivier *et al*, 1997; Ollivier *et al*, 1998; Sowers, 1995; Sprenger *et al*, 2000; Wasserfallen *et al*, 2000; Whitman *et al*, 1992; Wilharm *et al*, 1991; Winter *et al*, 1984; Zellner *et al*, 1999; Zellner *et al*, 1998; Zellner *et al*, 1990).

certain alcohols as the electron donors (Whitman *et al*, 1992). Among the 93 species in table 1.5, 67% are hydrogenotrophs. Of the hydrogenotrophic species 63% can also utilise formate, 18% alcohols and 31% are limited to hydrogen as their only electron donor. In addition, two species grow strictly by methanol reduction with hydrogen as the electron donor.

In the second group, the methylotrophs, the energy substrate is one of a variety of methyl-containing C-1 compounds. Usually these compounds are disproportionated. Some molecules of the substrate are oxidised to CO_2. The electron acceptors are the remaining methyl groups, which are reduced directly to methane (Whitman *et al*, 1992). In Table 1.5, 23% of species are methylotrophs. All the methylotrophic species belong to a single family, the *Methanosarcinaceae*.

Acetate is the major substrate of the third group. In this group methanogenesis proceeds by an aceticlastic reaction, in which the methyl carbon of acetate is reduced to methane and the carboxyl carbon is oxidised to CO_2 (Whitman *et al*, 1992). The ability to catabolize acetate is restricted to the genera *Methanosarcina, Methanosaeta* and *Methanothrix*. All species of the last two genera are strict acetotrophs, whereas species of *Methanosarcina* can also utilise methyl compounds. Just 9% of species in Table 1.5 are acetotrophs. Although the reduction of acetate to methane is limited to only a few described species, it has been estimated that two-thirds of the methane in nature originates from the methyl group of acetate (Ferry, 1992).

Under standard conditions the free energy change ($\Delta G^{\circ\prime}$) associated with the reduction of CO_2 with H_2 to CH_4 is $-131 kJ\ mol^{-1}$. However, in methanogenic

ecosystems, the H_2 partial pressure is generally 10Pa or less, so the free energy change associated with CO_2 reduction is only about –30kJ mol^{-1}. ATP generation requires more than 50kJ mol^{-1}, therefore less than 1 mol of ATP can be generated per mol of CH_4 formed (Thauer, 1997). Methanogenesis from other substrates generates even less energy (Table 1.6). The synthesis of ATP in methanogens is driven by ion gradients across the membrane, rather than by substrate-level phosphorylation (Schafer *et al*, 1999).

Table 1.6 Reactions and standard changes in free energies for methanogenesis.[a]

Reaction	$\Delta G^{\circ\prime}$ (kJ mol^{-1} of methane)
4 H_2 + CO_2 → CH_4 + 2 H_2O	- 135.6
4 Formate → CH_4 + 3 CO_2 + 2 H_2O	-130.1
4 2-Propanol + CO_2 → CH_4 + 4 Acetone + 2 H_2O[b]	- 36.5
2 Ethanol + CO_2 → CH_4 + 2 Acetate[c]	- 116.3
Methanol + H_2 → CH_4 + H_2O	- 112.5
4 Methanol → 3 CH_4 + CO_2 + 2 H_2O	- 104.9
4 Methylamine + 2 H_2O → 3 CH_4 + CO_2 + 4 NH_4^+	- 75.0
2 Dimethylamine + 2 H_2O → 3 CH_4 + CO_2 + 2 NH_4^+	- 73.2
4 Trimethylamine + 6 H_2O → 9 CH_4 + 3 CO_2 + 4 NH_4^+	- 74.3
2 Dimethylsulfide + 2 H_2O → 3 CH_4 + CO_2 + H_2S	- 73.8
Acetate → CH_4 + CO_2	- 31.0

[a] The standard changes in free energies were calculated from the free energy of formation of the most abundant ionic species at neutral pH. Thus, "CO_2" is HCO_3^- + H^+ and formate is $HCOO^-$ + H^+.
[b] Other secondary alcohols utilised, include, 2-butanol, 1,3-butanediol, and cyclopentanol.
[c] Other primary alcohols utilised, include, 1-propanol and 1-butanol.
Reproduced from Whitman *et al* (1992).

All methanogens share the same catabolic pathway for methane generation from the various substrates (Figure 1.7). The only differences being in the initial steps that convert the different substrates to intermediates of the pathway, and the direction in which the pathway operates. During methane formation from H_2 plus CO_2, reactions 1 to 5 (Figure 1.7) proceed in the direction of CO_2 reduction. The methyl groups of

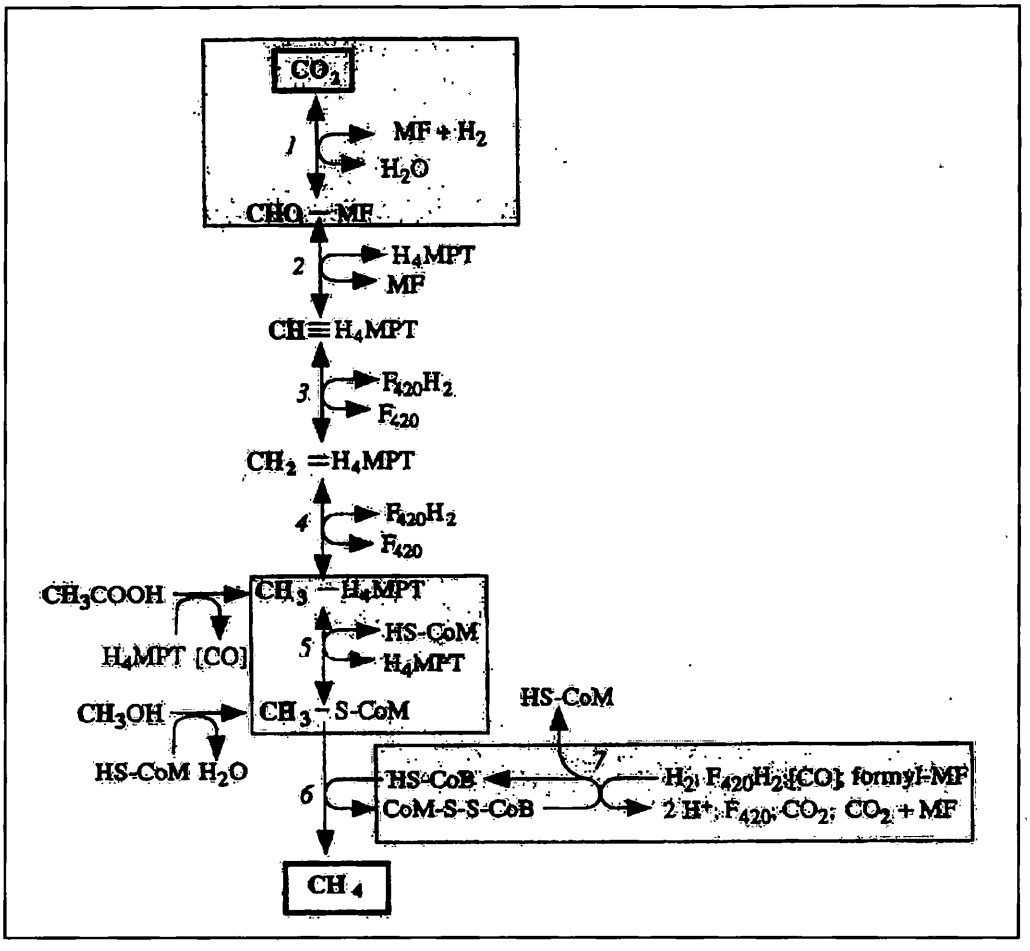


Figure 1.7 Pathways of methanogenesis.
Reactions involved in energy conservation are boxed. F_{420}, oxidised form of coenzyme F_{420}; $F_{420}H_2$, reduced form of F_{420}; HS-CoM, CoM (2-mercaptoethanesulfonate); HS-CoB, CoB (7-mercaptoheptanoylthreonine phosphate); CoM-S-S-CoB, heterodisulfide of HS-CoM and HS-CoB. Enzymes: 1, formyl-MF dehydrogenase; 2, formyl-MF:H_4 MPT formyltransferase and methenyl-H_4 MPT cyclohydrolase; 3, F_{420}-dependent methylene-H_4 MPT dehydrogenase; 4, F_{420} - dependent methylene-H_4 MPT reductase; 5, methyl-H_4 MPT:CoM-methyltransferase; 6, methyl-CoM reductase; 7, heterodisulfide reductase system (different electron donor systems are indicated). Reproduced from Schafer *et al* (1999).

methanol and acetate enter the central pathway at the level of H4 MPT. During methanogenesis from methanol, one-fourth of the methanol is oxidised to CO_2 by the reversal of reactions 1 to 5; the six reducing equivalents gained are used to reduce 3 mol of methanol to methane. During methanogenesis from acetate, the carboxyl group is oxidised to CO_2 and the electrons gained are used to reduce the methyl group

to acetate (Schafer *et al*, 1999). The reduction of methyl-CoM (reactions 6 and 7) is common to all methanogenic substrates. A number of unique coenzymes, some of which are found exclusively in methanogens are required for methanogenesis. The reactions involving these coenzymes and the enzymes that catalyse them may therefore also be unique to methanogens. These unique properties of methanogens provide a means of detecting and identifying methanogens in the environment.

1.4.5.2 Habitats of methanogens

A second unifying feature of methanogens is their extreme sensitivity to oxygen. For instance, the half-time for survival of one species of *Methanosarcina* is reported to be only four minutes in air-equilibrated medium (Whitman *et al*, 1992). Hence methanogens are generally found only in anoxic environments such as freshwater and marine sediments, peat bogs, anoxic rice field soils, anaerobic sewage digesters and deep-sea hydrothermal vents. However, methanogens have been isolated from environments where oxygen is present occasionally or at low levels, such as oral cavities and the hindgut of termites (Ferrari *et al*, 1994; Leadbetter and Breznak, 1996). In these environments, methanogens may be protected from oxygen by living in the centre of bacterial aggregates or biofilms, in which the aerobic bacteria consume the oxygen creating an anoxic microenvironment in the centre of the aggregate. Methanogens have also been detected in oxic soils. When soil samples were made anaerobic, methanogenic activity resumed after a lag phase (Peters & Conrad, 1995). Finlay & Fenchel (1991) demonstrated the presence of symbiotic methanogens living in the anaerobic ciliate, *Metopus palaeformis*, isolated from landfill material. They showed that the symbiotic methanogens survived for several days inside the ciliate host when exposed to atmospheric oxygen.

Methanogens have been isolated from environments at a wide range of temperatures. The majority of known methanogens are mesophilic. Approximately 60% of the species listed in Table 1.5 grow optimally at mesophilic temperatures (30-45°C). Methanogens have also been described with optimum growth temperatures in the thermophilic range (55-75°C) and hyperthermophilic range (>75°C). The majority of thermophilic species have been isolated from thermophilic waste digesters, as well as thermal marine and lake sediments (Table 1.5). Hyperthermophilic species with optimum growth temperatures between 80 and 98°C have been isolated from geothermally heated marine sediments and hydrothermal vents at a range of depths, as well as solfataric hot springs and mud (Table 1.5) (Kurr *et al*, 1991). The majority of hyperthermophilic species belong to the order *Methanococcales*. Two hyperthermophilic species, *Methanothermus fervidus* and *Methanothermus sociabilis* belong to the order *Methanobacteriales*, and a single species, *Methanopyrus kandleri* has been ascribed to a separate order, *Methanopyrales*. The majority of described thermophilic species belong to two genera, *Methanobacterium* and *Methanothermobacter* within the order *Methanobacteriales*.

Only a few psychrophilic/psychrotrophic species have been described. All of these species belong to the order Methanomicrobiales. Two species have been isolated from Ace Lake in Antarctica, *Methanococcoides burtonii* and *Methanogenium frigidum* (Franzmann *et al*, 1997; Franzmann *et al*, 1992). *M. burtonii* is a methylotrophic methanogen with an optimum growth temperature of 23.4°C and a maximum of 29.5°C, whereas *M. frigidum* grows by CO_2 reduction and has an optimum growth temperature of 15°C and a maximum of 18-20°C.

Most methanogens grow optimally around neutral pH, between 6.5 and 7.5, though acidophilic and alkaliphilic species have been described (Table 1.5). Examples of acidophilic methanogens include *Methanobacterium espanolae* and *Methanococcus igneus*, and alkaliphiles include *Methanohalophilus oregonense*, *Methanohalophilus zhilinae* and several species of *Methanobacterium*. Methanogens can also be found in environments with a wide range of salinities from freshwater to salt lagoon sediment. The most extreme example is *Methanohalobium evestigatum*, which will grow with salinity up to 5.1M NaCl (Wilharm *et al*, 1991).

1.4.5.3 Taxonomy of methanogens

The third distinctive feature of methanogens is that they are *Archaea*. The domain *Archaea* contains two kingdoms, the *Crenarchaeota* (comprising the extremely thermophilic *Archaea* of the genera *Pyrodictium* and *Thermoproteus*) and the *Euryarchaeota* (encompassing the *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Thermococcales* and the extreme halophiles) (Woese *et al*, 1990). Although there is still debate over the three domain system proposed by Woese *et al* (Embley *et al*, 1994), the methanogens and other *Archaea* do share some properties that distinguish them from the microorganisms of the domains *Bacteria* and *Eucarya*. Some of these properties are listed below:

- Capability of extreme thermophily in some groups.
- Lipids composed of glycerol ethers of isoprenoids and tetraethers are common.
- Stereochemistry of lipids is 2,3-*sn* glycerol.
- Cell walls composed of protein, glycoprotein, or pseudomurein; murein is absent.
- Antibiotic sensitivity differs from that of *Bacteria*.

- Unique modes of energy metabolism in some groups; i.e., bacteriorhodopsin-driven photosynthesis, methanogenesis.

(Whitman *et al*, 1992)

Methanogens are different from the other *Archaea* so far described because they are abundant in environments of moderate temperature, pH and salinity. Also, they are distinct from other *Archaea* due to their methanogenic metabolism and the possession of the unique coenzymes essential for methane synthesis (Whitman *et al*, 1992).

The methanogenic *Archaea*, like most bacteria, were originally classified on the basis of cell morphology (Balch *et al*, 1979). The taxonomy of methanogens was extensively revised in the light of new information based on comparative studies of 16S rRNA oligonucleotide sequences, membrane lipid composition, and antigenic fingerprinting data (Balch *et al*, 1979; Fox *et al*, 1977; Garcia, 1990). The taxonomy of methanogens and the positioning of novel isolates within the current taxonomy is now based largely on 16S rRNA sequence data. Though, additional characteristics including morphology, nutritional versatility, growth temperature, cell wall structure, G+C content of chromosomal DNA, antigenic relationships, analysis of unique enzymes and cofactors, and sequence data of other genes, are also used in the process of identification and classification (Garcia *et al*, 2000; Wasserfallen *et al*, 2000; Whitman *et al*, 1992).

The taxonomy of the methanogenic *Archaea* is constantly changing. In addition to the described species, there are numerous strains that have not been characterised sufficiently to determine if they constitute a new species. Also, new species are being

discovered all the time. As new information becomes available changes to the taxonomy are proposed, but it may take years for these changes to become accepted and validated. Boone *et al*, (1993) proposed a number of significant changes to methanogen taxonomy, including: 1) the creation of two new orders, *Methanosarcinales* (encompassing all the genera of the family *Methanosarcinaceae*) and *Methanopyrales* (to include a single species, *Methanopyrus kandleri*); 2) the creation of a new family *Methanosaetaceae* within the *Methanosarcinales*; 3) the reorganisation of the order *Methanococcales* (to include two families and four genera); 4) a new family *Methanospirillaceae* and 5) three new genera, *Methanothermobacter, Methanofolis* and *Methanosalsus*. Some of these changes such as the creation of a new genus, *Methanothermobacter* have been accepted and validated (Garcia *et al*, 2000; Wasserfallen *et al*, 2000). The most recent review of methanogen taxonomy (Garcia *et al*, 2000) summarised the taxonomic status of the changes proposed by Boone *et al*, (1993). In this thesis, I have used as far as possible only validated taxa names. The current taxonomy of methanogens is shown in Table 1.5 and the phylogeny of methanogens based on a Fitch distance matrix tree of the 16S rRNA gene is presented in Figure 1.8.

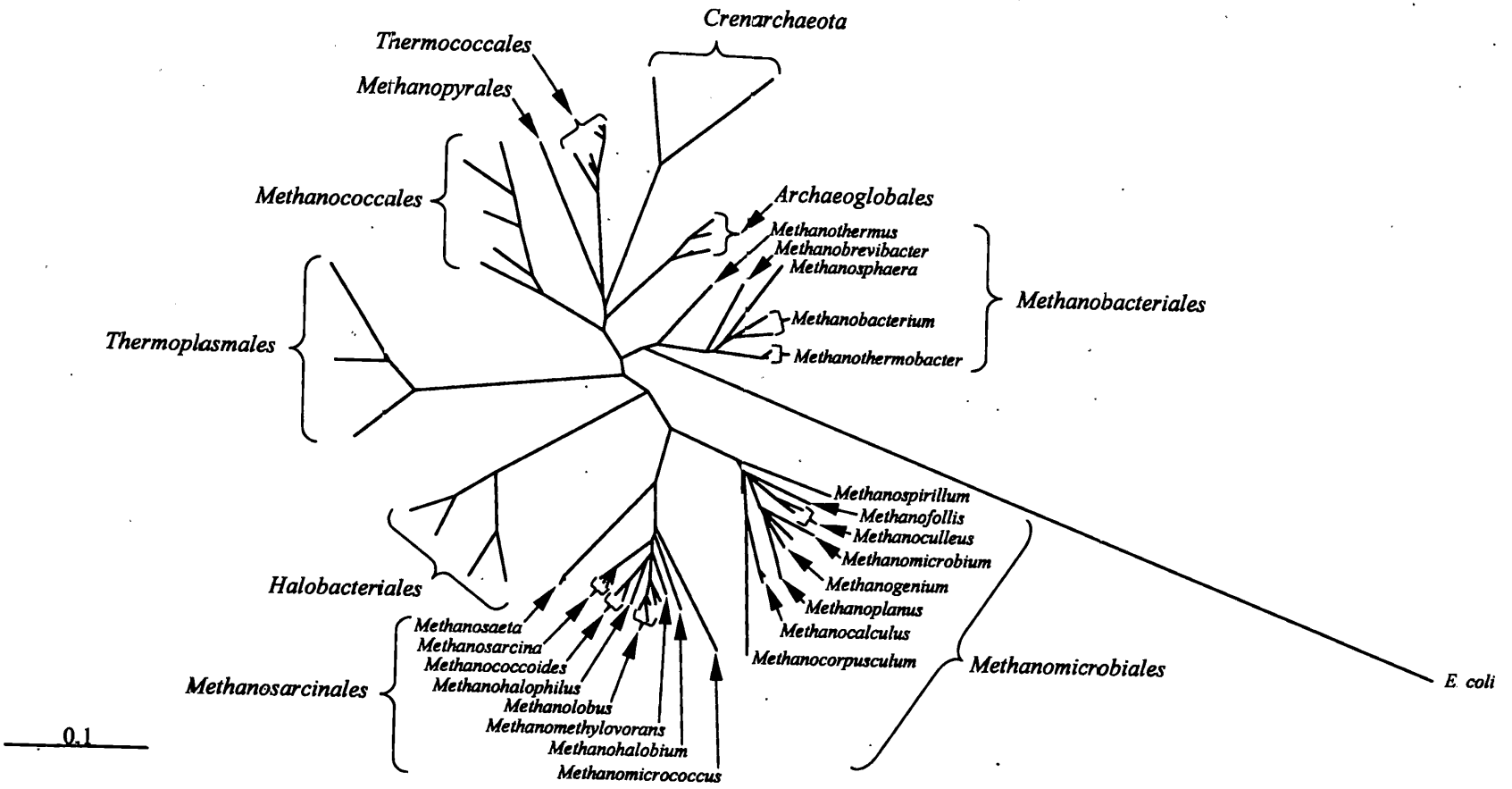


Figure 1.8 Phylogeny of methanogens, domain *Archaea*, based on 16S rDNA.
Non-methanogens are included to illustrate the relationship of the methanogens to the rest of the *Euryarchaeota*, the *Crenarchaeota* and the *Bacteria*. 16S rRNA gene sequences were obtained from GenBank. Sequences were aligned using PILEUP in Wisconsin Package 10.1 (Genetics Computer Group). The phylogenetic analysis was performed by DNADIST and FITCH in PHYLIP 3.57c (Felsenstein, 1995). The tree was viewed using TREEVIEW 1.6.1 (Page, 1996).

1.5 Investigating the microbial ecology of landfills

In the previous sections I have reviewed what is known about the biological process of waste decomposition in landfills and the microorganisms that are known or believed to be involved. The objective of this section is to describe some of the techniques that may be used to investigate the microorganisms of the landfill ecosystem.

1.5.1 Sample collection

Obtaining representative samples from landfills is the first problem encountered when setting out to investigate landfill microbiology. The nature of the wastes buried in landfills means that they are extremely heterogeneous and as a result it is probably not possible to obtain truly representative samples from landfills (Barlaz, 1997). The objective of a study will determine the optimum sample collection strategy. If the aim is to survey the complete landfill microbial community, then it is important to obtain representative samples. On the other hand, if the aim is to detect novel microorganism in landfill or isolate and characterise representatives of a particular group of microorganisms from landfill, then obtaining samples representative of the entire landfill microbial community is not necessary.

If representative samples are desired, Barlaz (1997) recommends obtaining multiple samples over a preselected grid pattern. Samples are commonly excavated either using construction equipment, such as a JCB fitted with a back-hoe, or using drilling equipment, such as bucket augers or cable-tool drills (Barlaz, 1997; Fredrickson & Phelps, 1997; Suflita *et al*, 1992; Westlake *et al*, 1991). Both types of drilling

equipment are suitable for obtaining samples from depths up to 100m. Barlaz (1997) recommends excavating large samples (about 1,000kg) and reducing them using quartering techniques to obtain as representative a sub-sample as possible. When culture-based techniques are to be used for microbiological analyses, there is often concern regarding sample exposure to air. Many of the microorganisms involved in decomposition are obligate anaerobes, and methanogens in particular are extremely sensitive to oxygen exposure. However, it has been shown that exposure of refuse samples, in an active state of methane production, to air for 2 to 4 hours, does not increase the time required for these samples to resume methane production, once replaced in an anaerobic system (Barlaz, 1997). In practice, samples are routinely placed either in a series of two oxygen-impermeable plastic bags, which are tightly sealed after removal of excess air, or they are placed in air-tight containers, which are flushed with nitrogen before sealing (Suflita *et al*, 1992; Westlake *et al*, 1991).

Sample collection regimes vary from one study to the next. Suflita *et al* (1992), whose objective was to obtain refuse that had been buried for various lengths of time, used a systematic sampling scheme to sample every major section of the Fresh Kills Landfill in New York. They used a bucket auger to drill 14 boreholes at various locations and collected a total of 47 samples at 3m depth intervals. Westlake *et al* (1991) used a bucket auger to drill boreholes at 2m intervals in a landfill test cell at the Brogborough Landfill, Bedfordshire, England. Samples were taken from depths of 5, 10 and 15m in each borehole. Sub-samples were used for determination of moisture content, pH, substrate pool size and measurement of methane production. Ladapo & Barlaz (1997) excavated a single sample of refuse using a hand auger, after removing about 2.1m of overlying soil. The sample was placed in a refuse bag

flushed with nitrogen for 30 minutes before being transported to the laboratory. The objective of the study was the isolation of acidophilic methanogens from landfill. Daly *et al* (2000) obtained samples of landfill leachate from seven landfills for detection of SRB by PCR and probing. Several samples of fresh leachate were collected from each site and pooled. These pooled samples were considered to be representative of each landfill site as a whole.

1.5.2 Sample processing

When performing a microbiological analysis of any environmental sample, the effects of sample processing should be considered, whether the analysis is culture-based or molecular. Excavated landfill samples may be subject to a number of different processing techniques before being analysed. Typically, these processes may include: hand-sorting to remove large or unusual objects, shredding to reduce the particle size, mixing or blending in sterile, anaerobic buffer to release the microorganisms from the solid material, filtering or centrifugation to remove larger solid particles. Sub-samples of the liquid fraction remaining from this process may be used to inoculate enrichment cultures, used in enumeration techniques or subjected to further processing for other analysis techniques. Westlake *et al* (1991) shredded 2kg sub-samples from excavated landfill samples, using a garden shredder under a blanket of nitrogen. Under anaerobic conditions, 25g samples of the shredded waste were placed into 100ml Wheaton bottles together with 25ml degassed, distilled water. Radiolabelled substrate diluted in 25ml degassed, distilled water was added to the bottles, which were sealed and incubated at 30°C. Ladapo and Barlaz (1997) prepared inocula for methanogenic enrichment cultures by blending 100g sub-samples of excavated landfill material in phosphate buffer in an autoclaved, N_2-sparged

blender. The resulting slurry was then hand-squeezed to produce a liquid inoculum. Prior to DNA extraction, Daly *et al* (2000) processed landfill leachate samples by centrifuging 1L samples of leachate (27,000×g, 40min) and resuspending the resulting pellets in 20ml of phosphate buffer. Aliquots (1.5ml) of the concentrated sample were centrifuged (22,000×g, 5min) and the pellets stored at –80°C. The frozen pellets were thawed on ice and resuspended in 200µl of sterile distilled water. DNA was extracted from the resuspended pellet. LloydJones and Lau (1998) extracted DNA from 0.5g samples of landfill cover soil.

Few studies have evaluated the effectiveness of sample processing techniques. Barlaz *et al* (1989) blended refuse samples in anaerobic phosphate buffer, then hand-squeezed the blended sample and used the resulting liquid as an inoculum. They evaluated the effect on cell extraction of prechilling the refuse at 4°C, multiple blendings and hand-squeezings, and the use of blended refuse prior to hand-squeezing. The additional treatments did not increase the most probable number (MPN) of cellulolytic bacteria above the population measured by blending followed by hand squeezing (Barlaz, 1997; Barlaz *et al*, 1989). Maule *et al* (1994) evaluated the efficiency of an alternative cell extraction technique. Duplicate 20g samples of refuse were homogenised in 180ml of phosphate buffer for 1 minute. This extraction procedure was repeated six times, and its efficiency was judged on the basis of the number of aerobic bacteria that would grow on tryptone soya agar at 35°C. It was reported that 93.4% of the cells that were extractable were extracted in two cycles of the extraction procedure.

1.5.3 Microbiological analysis

The techniques that may be used to characterise the microbiology of a landfill sample include enumeration methods, enzyme assays, measurement of methane production rate, and molecular techniques such as antigenic fingerprinting, lipid analysis and nucleic acid-based techniques.

1.5.3.1 Enumeration

A number of methods have been used for the enumeration of landfill microorganisms including acridine orange direct counts (AODC), MPNs, agar plate counts and roll tubes. AODC can be performed directly on landfill samples and provide a measurement of the total number of bacteria regardless of their viability (Palmisano *et al*, 1993). The other methods are culture dependent and therefore enumerate only viable, culturable microorganisms. Comparison of AODC with culture dependent methods may provide some indication of the proportion of microorganisms in landfill that are not culturable under laboratory conditions. Indeed, it has been estimated that we cannot culture the vast majority (>99%) of naturally occurring microbes using standard techniques (Hugenholtz & Pace, 1996).

The functional groups of microorganisms may be enumerated by using culture media containing different carbon sources, to select for those organisms best able to utilise a particular carbon source, and/or using antibiotics or other compounds that specifically inhibit a particular group of microorganisms. In MPN enumeration, the growth of cellulose degraders was detected by the visible disappearance of ball-milled Whatman No. 1 filter paper (Barlaz, 1997). Mackie & Bryant (1981) enumerated syntrophic acetogenic bacteria on the basis of conversion of butyrate or propionate to acetate and

hydrogen and subsequent conversion of the hydrogen to methane by a pure culture of *Methanospirillum hungatei*. Positive tubes were counted as those that contained a methane concentration higher than that in control tubes lacking butyrate or propionate. Homoacetogenic bacteria were enumerated in two soil samples by a colourimetric MPN assay (Harriott & Frazier, 1997). This assay utilised the ability of homoacetogens to anaerobically-*O*-demethylate a methoxylated aromatic substrate (vanillate) to produce a coloured product. The growth of methanogens in MPN assays may be detected by the production of methane. Bacteria that may out grow methanogens due to their faster growth rate may be selectively inhibited by the addition of antibiotics such as penicillin G or kanamycin to the growth medium (Whitman *et al*, 1992). Methanogenic colonies growing in roll tubes may be distinguished from non-methanogenic bacteria by taking advantage of the fluorescent pigment, cofactor F_{420}, which is abundant in methanogens and fluoresces when exposed to ultra-violet light. Kataoka *et al* (1991) described a method for counting methanogenic colonies in roll tubes using an epifluorescence microscope.

1.5.3.2 Enzyme assays and other direct measures of microbial activity

Assays of specific enzyme activities can be used to measure the activity of functional groups of microorganisms in environmental samples. Cellulase, protease, amylase and lipase activities have been measured in landfill samples (Jones and Grainger, 1983; Palmisano and Barlaz, 1996). Enzyme assays have limitations relating to the efficiency of enzyme recovery and the inactivation of enzymes by proteases present in the samples (Barlaz, 1997). Jones and Grainger (1983) observed differences in the recovery of a commercial cellulase added to sterile and non-sterile refuse samples. Upon extraction, the cellulase was fully recovered from the sterile refuse, but only a

fixed amount of enzyme activity was measured in the non-sterile refuse regardless of the amount of enzyme added. The authors suggested that the enzyme could have been deactivated by proteases. It has also been suggested that low measures of cellulase activity may be because one or more of the enzymes responsible for cellulose hydrolysis is membrane bound and not extracted (Barlaz, 1997).

Two other methods that have been used to assess microbial activity in landfill samples are mineralization of ^{14}C-cellulose and quantitation of cofactor F_{420}. Mineralization of ^{14}C-cellulose to $^{14}CO_2$ and $^{14}CH_4$ provides a measure of the activity of all the trophic groups involved in cellulose conversion to methane (Barlaz, 1997). Quantitation of cofactor F_{420} by high performance liquid chromatography (HPLC) can provide a measure of methanogenic biomass. However, the accuracy of the method is limited because F_{420} from environmental samples may include F_{420} extracted from non-viable cells and extracellular F_{420}. In addition, F_{420} concentration varies between species and with growth conditions (Peck & Archer, 1989). All these assays provide a measure of the activity of the microbial community or specific microbial groups. However, they do not give any information on the composition of the groups or the identity of individual species.

1.5.3.3 Molecular techniques

The limitations of culture dependent and activity-based methods as described above have stimulated the development of a number of molecular techniques for the detection and identification of microorganisms in the environment.

The extraction and quantitation of cell membrane lipids has been used to enumerate and identify a range of bacteria in natural environments (Peck and Archer, 1989). *Archaea* possess membrane lipids that are distinct from those of *Bacteria*, and variations in the lipid profile of methanogens permits identification to the family or genus level (Koga *et al*, 1998). However, the effects of substrate and growth conditions on lipid profiles may limit the usefulness of this technique for identifying methanogens in environmental samples (Peck and Archer, 1989).

Serological techniques based upon antigen/antibody interactions have been used extensively for the identification of many different bacteria (Conway de Macario *et al*, 1982; Macario & Conway de Macario, 1985). A number of studies have used immunological procedures such as enzyme-linked immunosorbent assay (ELISA) for the characterisation of methanogens in anearobic digesters (Archer, 1984; Bryniok & Trosch, 1989; Gorris *et al*, 1987; Kemp *et al*, 1988; Macario and Conway de Macario, 1985; Sorensen & Ahring, 1997). Fielding *et al* (1988) characterised seven methanogenic isolates from landfill samples by using antigenic fingerprinting to compare the relatedness of the isolates to a reference methanogen culture collection. Polyclonal and monoclonal antibodies have been demonstrated that allow the identification of methanogens in environmental samples down to level of individual strains (Sorensen and Ahring, 1997). Limitations of these techniques include: the fact that only those species probed for may be detected, making it possible to miss other species; novel species may not be detected if they do not cross react with existing antisera; species must be available in pure culture to allow generation of antibodies; and cell antigenicity may vary depending on growth conditions (Peck and Archer, 1989).

Techniques based on nucleic acids have come to dominate studies of microbial ecology. The variety of nucleic acid technologies and their application to the landfill environment will be described in the following section.

1.5.4 Nucleic acid technologies

The range of methods based on nucleic acids and their applications is growing all the time. In the field of microbial ecology, DNA and RNA based methods have been applied to problems such as: the identification of novel isolates; the detection of microorganisms in diverse environments; the localisation of microorganisms within biofilms, microbial aggregates and protozoa; quantitation of microbial groups and species; measurement of microbial community diversity; and the monitoring of changes in community structure. The principal advantages of nucleic acid (NA) techniques are that they are not hindered by our inability to culture the majority of microorganisms in the environment, and DNA, unlike other cell components such as membrane lipids or cell surface antigens, is not subject to variation due to growth conditions. The first step for any NA technique is to make the genetic material of the target organism/s accessible. For the majority of procedures this involves some form of DNA extraction and purification.

1.5.4.1 DNA extraction

Many different protocols have been described for the extraction of NA from environmental samples, which could be applied to landfill and leachate. In general, the methods take one of two approaches: 1) the cells are separated from the sample matrix before cell lysis and NA extraction; 2) the cells are lysed directly in the

presence of the sample matrix and the DNA is recovered (Wheeler Alm & Stahl, 1996). The two approaches have their advantages and disadvantages as described below. The methods of cell lysis and NA purification vary between methods, but generally follow the same pattern.

General outline of nucleic acid extraction procedures:

1) Cell lysis by mechanical, chemical, enzymatic or a combination method.
2) Several purification steps to separate NA from other cell components and matrix particles.
3) Further purification of NA.

When considering methods of NA extraction from environmental samples there are several concerns that should be addressed:

- What fraction of total NA is recovered from the environmental matrix?
- Is the recovered fraction representative?
- Integrity of recovered NA?
- Purity of recovered NA?

There are several factors that can affect the recovery of NA from an environmental matrix such as landfill. Bacteria may be bound to soil particles by extracellular bacterial polymers, humic colloids, electrostatic forces, hydrogen bonding and other surface interactions (Torsvik, 1995). The extent of binding varies for different soil types and different bacteria. Bacteria may also be physically entrapped in soil aggregates. The binding of bacteria to soil particles is of greater concern with indirect extraction methods, in which the microbial cells are first separated from the soil or

other environmental matrix before lysing. In direct extraction methods the cells are lysed in the presence of the environmental matrix, so binding of cells to matrix particles should not be a problem. However, the released NA may bind to the matrix. Ogram *et al* (1988) found that sediments containing significant amounts of montmorillonite clay could adsorb greater than 200μg of DNA per gram of sediment. The extent of DNA adsorption to matrix particles is affected by the mineralogy of the sorbent, pH, ionic strength and length of DNA (Ogram *et al*, 1988).

The recovered NA fraction may not be representative. For example, a population resistant to breakage may be under-represented, while an exceptionally easy-to-break microorganism may be over-represented (Stahl, 1997). The representativeness of the recovered NA fraction from different extraction procedures can be monitored by comparing the total rRNA recovery, as determined by hybridisation with a universal probe, to the recovery of rRNA from specific target groups, measured with group specific probes (Stahl, 1997).

The integrity of the recovered NA is affected by the lysis technique employed. Mechanical disruption procedures, such as bead-beating, freeze-thawing, sonication and microwave heat treatment, can shear DNA (Wheeler Alm and Stahl, 1996). Highly sheared DNA may be suitable for some purposes, such as PCR of short fragments, while high molecular weight DNA is required for others, such as hybridisation, cloning or PCR of whole genes (Wheeler Alm and Stahl, 1996). Less disruptive lysis methods, such as chemical or enzymatic lysis, may be used for recovery of high molecular weight DNA. However, this may lead to under-representation of harder-to-break organisms. For example, methanogens are not

susceptible to lysis with lysozyme since they lack peptidoglycan in their cell walls (Rinker & Evans, 1991). The integrity of NA may also be affected by degradation from nucleases released during cell lysis. This presents more of a problem when working with RNA than DNA, particularly the unstable messenger RNA (mRNA). For this reason methods that rapidly inactivate nucleases or separate the NA from the nucleases are favoured. The integrity of extracted NA may be determined by gel electrophoresis.

The importance of the purity of the recovered NA depends on the use intended. Inhibition of restriction enzyme digestion and PCR of DNA extracted from sediments, has been attributed to contamination with humic substances (Wheeler Alm and Stahl, 1996). Humic substances result from the progressive cross-linking of partly degraded organic compounds to form heterogeneous high molecular weight (500 to >250,000 Daltons) organic molecules (Wheeler Alm and Stahl, 1996). Humic substances are ubiquitous in soils and sediments and tend to co-purify with NA. However, extraction methods are readily available now, which eliminate these contaminants, for example the methods described by (Saano & Lindstrom, 1995; van Elsas & Smalla, 1995).

1.5.4.2 PCR – success and failure

The polymerase chain reaction (PCR) forms the basis of many of the methods used to analyse nucleic acids recovered from the environment. PCR amplification offers the advantages of specificity and sensitivity. As few as 10 copies of a gene per gram of soil can be detected, as compared to 10^4 copies by DNA-DNA hybridisation (Picard *et al*, 1996). DNA extracted from the environment can be used directly as a template for

PCR amplification, while RNA is first reverse transcribed to cDNA by a reverse transcriptase. The cDNA is then used as a template for PCR.

The principles and details of the PCR have been the subject of numerous books and articles, for example the book by Erlich (1989). Briefly, PCR amplification involves the repeated cyclic enzymatic extension of primers at two opposite ends of a DNA template, resulting in the generation of numerous copies of this template (van Elsas & Wolters, 1995). The amplification cycle, composed of template denaturing, primer annealing and extension steps, is achieved by concerted changes in reaction temperature. Thermostable DNA polymerases are used that maintain activity through repeated heating to 94-95°C.

Strategies for successful PCR

The optimisation of any PCR, in terms of specificity, sensitivity, fidelity and reproducibility, is affected by a number of factors. These factors include: the primers; the temperature and duration of the denaturing, annealing and extension steps; the number of cycles; the Mg^{2+} concentration; the quality and concentration of the template; and the presence of inhibitors. In general, it is recommended that primers are 18 to 25 bases in length, have a G+C content of around 50%, are free of complementarity within and between primers, and have cytosine or guanine bases in the last two positions at the 3' end. Group specific primers can be designed that bind to conserved regions of genes. A certain amount of degeneracy can be incorporated into primers, but not close to the 3' end.

A number of strategies can be employed to improve the specificity of PCR amplifications. 'Hot start', in which one of the reaction components is left out until the reaction reaches denaturing temperature, avoids the generation of non-specific products before the first cycle. Touch-down PCR attempts to limit initial priming to high-fidelity (primer-target) annealing by using restrictive (high) initial primer annealing temperatures, which are progressively lowered in the following cycles to allow for more efficient subsequent amplification cycles (van Elsas and Wolters, 1995). In nested PCR two separate reactions are set up. The second reaction uses the product of the first as a template and primers that are internal to the primers used in the first reaction.

The inhibition of PCR amplification by contaminants co-purified with the NA can sometimes be overcome by the dilution of the template solution, by the addition of PCR facilitators such as formamide, bovine serum albumin or DMSO, or by the use of different polymerases. Some commercial DNA polymerases, such as Platinum Pfx DNA Polymerase (Gibco BRL), come with enhancer solutions that can be used to facilitate amplification or improve the yield or specificity. A recent review by Wilson (1997) covers extensively the inhibition and facilitation of PCR amplification of NA extracted from a range of clinical, food and environmental sources.

Pitfalls of multi-template PCR amplification

The PCR is commonly used for the amplification of genes from the total microbial community or sub-sections of the community from environmental samples. The information gained from the analysis of the amplified genes is used to make inferences about the diversity and abundance of microorganisms in the environment.

However, this strategy has a number of pitfalls that may lead to incorrect conclusions about the microbial community.

Bias in PCR amplifications has been observed by a number of workers. Reysenbach *et al* (1992) observed that when 16S rRNA genes were amplified from template mixtures containing equal numbers of rRNA genes from two *Archaea* and one yeast, the yeast gene was amplified preferentially. Mutter & Boynton (1995) found that amplification of alleles of a human androgen receptor gene were biased towards amplification of the lower molecular weight allele when the template concentration was low, the template was damaged or monovalent salts were present. They concluded that differences in secondary structure stability of the two alleles leading to differences in amplification efficiency was one of the major causes of the observed PCR bias. Suzuki & Giovannoni (1996) observed bias towards a 1:1 mixture of genes in the final products, regardless of the initial proportions of the templates. They also observed that the bias was strongly dependent on the number of cycles of replication and did not occur with a different primer pair. They concluded that the bias was caused by the progressive inhibition of primer-template hybrid formation by reannealing of product molecules at high product concentrations. The template with the higher initial concentration in the starting mixture reaches inhibitory concentrations sooner while the second template continues to undergo amplification efficiently, and thereby the original difference in concentrations decreases until a 1:1 ratio is achieved (Suzuki and Giovannoni, 1996). Chandler *et al* (1997) found that template concentration had a significant effect on the diversity and abundance of RFLP patterns detected after amplification of total community 16S rDNA from sediment samples. They proposed that very low template concentrations in the PCR

generate random fluctuations in priming efficiency. Polz & Cavanaugh (1998) found that when PCR amplification was performed with degenerate primers, templates with GC-rich permutations of the primer binding site were reproducibly overamplified compared to templates with AT-rich priming sites.

A second PCR phenomenon, that of chimera formation may lead to the overestimation of community diversity or to the description of non-existent species. Chimeric PCR products are composed of regions from two or more template molecules. Chimera formation has been demonstrated when the PCR is used to retrieve small sub-unit (SSU) rRNA sequences from natural microbial communities (Barns *et al*, 1994; Choi *et al*, 1994; Moyer *et al*, 1994). Chimeric or recombinant molecules have also been formed during PCR amplification of other genes, namely the HIV1 *tat* and *env* genes and the gene encoding cow lysozyme (Meyermans *et al*, 1990; Paabo *et al*, 1990). A number of factors have been implicated in the formation of chimeras. These include damaged template, premature termination of primer extension and templates consisting of complex mixtures of homologous sequences (Meyermans *et al*, 1990; Paabo *et al*, 1990; Wang & Wang, 1996).

Chimeric SSU rRNA sequences may be detected by analysing their secondary structure for abnormalities (Kopczynski *et al*, 1994). However, this method is not applicable to functional gene sequences. Chimeric functional gene sequences may be detected by demonstrating that separate domains of an unknown sequence are identical to different known sequences (Robison-Cox *et al*, 1995). Alternatively, chimeras may be detected by comparing the phylogenetic affiliations of different sequence domains. A computer program, CHIMERA_CHECK, is available at the

Ribosomal Database Project (RDB), which detects chimeras by determining nearest neighbours in variable unaligned sequence domains (Robison-Cox *et al*, 1995). However, this program is mainly suited to detecting chimeric SSU rRNA sequences.

From all the research on PCR bias and chimera formation a number of conclusions can be drawn. To minimise PCR bias and chimera formation PCR amplifications should be performed with the least possible number of amplification cycles, longer elongation times, high molecular weight intact template DNA, pure DNA free of contaminants, and non-degenerate primers (Meyermans *et al*, 1990; Mutter and Boynton, 1995; Paabo *et al*, 1990; Polz and Cavanaugh, 1998; Suzuki and Giovannoni, 1996; Wang and Wang, 1996). The researchers fail to agree on the best template concentration to minimise bias and chimeras. Meyermans *et al* (1990) concluded that recombinant sequences should not be problematic if lower template concentrations are used, while Polz and Cavanaugh (1998) found that bias was reduced considerably by using high template concentrations and mixing replicate reactions. Chandler *et al* (1997) proposed that several different template dilutions should be utilised during PCR when maximum diversity in clone libraries is desired. Finally, Reysenbach *et al* (1992) found that the addition of acetamide to PCRs minimised non-specific annealing and prevented preferential amplification.

1.5.4.3 Nucleic acid techniques for characterising microbial communities

A wide range of nucleic acid based techniques have been adapted for the characterisation of microbial communities without cultivation. Some of these techniques are summarised in Table 1.7. The techniques described in Table 1.7 have been applied to samples from a diverse range of habitats, such as rice-field soils,

anaerobic digesters and deep-sea sediments. The different methods are frequently used in combination (Figure 1.9). For example, denaturing gradient gel electrophoresis (DGGE) has been used to provide information on community diversity, in combination with probing or direct sequence analysis to identify the bands on the denaturing gradient gels (Teske *et al*, 1996).

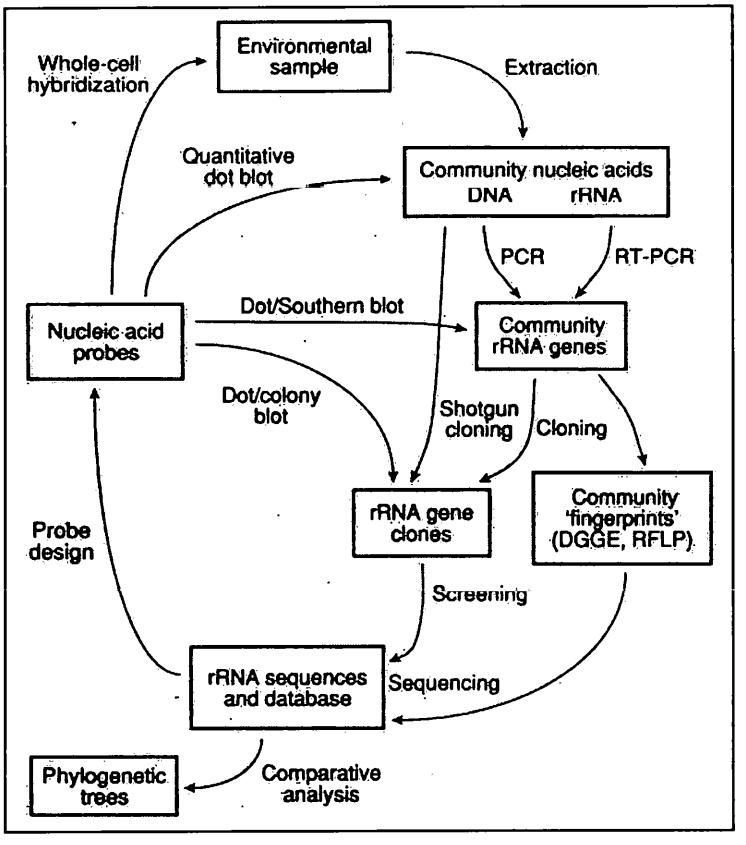


Figure 1.9 Strategies for characterising microbial communities without cultivation. Reproduced from Hugenholtz and Pace (1996).

There are only a few examples of nucleic acid techniques being used to characterise microbial communities in landfills. Maule *et al* (1994) were among the first to apply DNA-based techniques to landfill samples. They prepared probes for 13 species of methanogens and hybridised them to PCR products generated from the methanogen gene encoding sub-unit A of methyl coenzyme M reductase (*mcrA* gene), to check for

each species in landfill leachate. Five species, *Methanoculleus bourgensis, Methanobacterium formicicum, Methanosarcina barkeri, Methanosphaera stadtmanae* and *Methanobrevibacter ruminantium*, were shown to be present. Silvey & Blackall (1995) used DGGE to examine the microbial community in leachate generated from laboratory-scale reactors containing MSW. They used a range of PCR primers to amplify different regions of the 16S rRNA gene. LloydJones and Lau (1998) used PCR amplification of 16S rDNA followed by cloning, sequencing and phylogenetic analysis to characterise the microbial community in soil samples from a landfill. Wise *et al* (1999) used primers specific for the 16S rDNA of methanotrophic bacteria. They used PCR, cloning, sequencing and phylogenetic analysis combined with DGGE and conventional culture-dependent techniques to characterise the methanotroph community and describe novel isolates from landfill soil samples. Finally, Daly *et al* (2000) used PCR amplification in combination with genus-specific and multi-genus group-specific probes to characterise sulfate-reducing bacteria in landfill leachate.

Table 1.7 Summary of nucleic acid techniques for characterising microbial communities without cultivation

Method	Basis	Application	Reference
Hybridisation methods			
Nucleic acid hybridisation (Probing)	These methods rely on the ability of single-stranded nucleotide sequences to anneal only to complementary or closely related sequences under pre-determined conditions. The conditions can be altered to adjust the specificity of the hybridisation. The probes can be oligonucleotides (18-25 mer), whole genes or entire genomes.	Probing encompasses a wide range of methods, which differ in the type of probe and the method of preparing the target nucleic acid. Probes can be designed to target specific species, phylogenetic groups or functional groups. Probing methods can be used for detection, identification, enumeration or localisation of target genes. Probe design relies on sequences of the target gene being available.	(Nakatsu & Forney, 1996; Stahl, 1997)
Whole-cell hybridisation	Labelled oligonucleotide probes are hybridised to fixed, permeablized cells. The target is usually rRNA because of its abundance, but other genes may be targeted. Fluorescent labels are used frequently, hence fluorescent in situ hybridisation (FISH).	Identification, enumeration, spatial distribution and assignment of gene sequences to cells in biofilms, aggregates and endosymbionts of protozoa.	(Amann, 1995)
Southern blotting, Restriction fragment length polymorphism (RFLP)	Total community DNA is digested with restriction endonucleases. The fragments are separated by gel electrophoresis and transferred to a membrane support for hybridisation with a labelled probe.	Provides a measure of community diversity. These methods are more often used to relate different microbial isolates on the basis of restriction fragment length polymorphism.	(Stahl, 1997)
Reverse sample genome probing (RSGP)	Genomic DNA from a range of reference organisms is immobilised on a membrane support, the reference panel. Environmental DNA is randomly labelled and hybridised to the reference panel. The whole genome probes hybridise only to identical or closely related genotypes.	The method allows identification of microorganisms in a sample in a single step, provided an identical or closely-related genome is present on the reference panel. A disadvantage of the method is that the sample microbial community is described only in terms of its culturable component.	(Stahl, 1997; Voordouw *et al*, 1991)

Table 1.7 (continued)

Method	Basis	Application	Reference
PCR methods			
PCR-RFLP	Genes or gene fragments are amplified by PCR with universal or group specific primers. The PCR products are cloned then digested with restriction endonucleases. The fragments are separated by gel electrophoresis.	The resulting banding patterns are used for identification or as a measure of diversity. For identification, banding patterns must first be matched with sequences or probing results.	(Moyer *et al*, 1994)
Terminal-RFLP (T-RFLP)	Fluorescently end labelled PCR products are digested with restriction endonucleases (normally 4bp cutters) and the fragments separated by electrophoresis with laser detection of the labelled fragments.	A rapid method to characterise microbial community structure. Also provides an indication of the relative proportion of each component of a population. Automation means high sample throughput is possible.	(Osborn *et al*, 2000)
Denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis (DGGE/TGGE)	PCR products of the same length, but different sequence are separated based on decreasing mobility of partially melted DNA molecules in polyacrylamide gels with linearly increasing gradient of urea and formamide (DGGE) or temperature (TGGE). Sequence variation between DNA molecules leads to different melting behaviour and hence mobility at a particular denaturant concentration or temperature.	A rapid method to characterise community structure and changes. Can be applied to the detection of different genes or sub-populations. Also, the method is amenable to semiquantitative assessments of activity, by comparing banding patterns derived from RNA and DNA.	(Muyzer *et al*, 1993; Stahl, 1997)
PCR-single strand conformational polymorphism (PCR-SSCP)	PCR products are generated then denatured. The single-stranded DNA molecules are separated in a polyacrylamide gel. Sequence variation between PCR products leads to variation in secondary structure or conformation of the single stranded molecules and hence different mobilities in the gel.	Similar to DGGE/TGGE. In a refinement of the method one strand of the PCR product is fluorescently labelled and electrophoresed on an automated DNA sequencer. The result from a microbial community is a pattern in which each peak can be correlated with the gene sequence of one microorganism.	(Clapp, 1999; Zumstein *et al*, 2000)
Taq nuclease assay or 5' nuclease assay (TNA)	The 5'→3' exonuclease activity of *Taq* DNA polymerase is used to cleave a fluorescent labelled oligonucleotide hybridised to the PCR template. A fluorescent 3' end label quenches the fluorescence of the 5' end label. The fluorescence of the 5' label is emitted when it is cleaved from the probe and the accumulation of fluorescence can be related to the initial copy number of the target genes.	Provides a rapid, automated quantitation of PCR target genes. The method relies on suitable primers and probes being available.	(Becker *et al*, 2000; Suzuki *et al*, 2000)

Table 1.7 (continued)

Method	Basis	Application	Reference
Quantitative-PCR (Q-PCR)	PCR products are quantified by reference to an external standard curve, an internal reference template, an internal competitive standard or by MPN methodology.	Allows highly sensitive quantitation of target genes.	(Jansson & Leser, 1996)
Reverse transcriptase-PCR (RT-PCR)	RNA is transcribed to cDNA by reverse transcriptase, then cDNA is amplified by PCR.	Provides a measure of transcription level of target genes and hence activity since active cells contain more RNA than dormant or dead cells.	(Selenska-Pobell, 1995)
In situ PCR/RT-PCR	Same as whole-cell hybridisation, except using PCR and/or RT-PCR to increase the sensitivity of detection.	Highly sensitive in situ detection of specific genes and gene transcripts in complex natural microbial communities.	(Chen *et al*, 1998)
Other methods			
Low molecular weight-rRNA typing (LMW-rRNA typing)	Low molecular weight 5S rRNA and tRNAs extracted from environmental samples are separated by high resolution gel electrophoresis to generate a community profile, which can be related to banding patterns from pure culture isolates.	Provides an all-encompassing and direct measure of community structure and change in structure without any bias as introduced by PCR and cloning. Individual species may be identified, but due to resolution limitations the method is most applicable to low complexity communities.	(Stahl, 1997)
DNA reassociation	Total community DNA is denatured then allowed to reassociate. The time required for 50% reassociation under defined conditions is proportional to the complexity (e.g., number of unique genomes) of the DNA.	Provides a measure of total community complexity without bias. Although this method has been applied to relatively few communities, the results are notable in that they suggest far greater diversity than anticipated.	(Stahl, 1997)
Direct sequence analysis	The nucleotide sequence of whole genes or gene fragments are determined. Gene sequences may be recovered from environmental samples by shotgun cloning, screening and sequencing; cDNA cloning and sequencing; or PCR amplification, cloning and sequencing. To avoid sequencing of redundant clones, clone libraries are screened usually by probing or restriction analysis.	Phylogenetic analysis can be used to determine the phylogenetic affiliations of sequenced genes. Or closely matching sequences in sequence databases can be identified using computer programs such as BLAST.	(Stahl, 1997)

1.6 Aims and objectives

The realisation that the microbial degradation of waste in landfills contributes to the production of landfill gas and leachate, provided an impetus for research in this area (Lawson, 1989a). It was hoped that a better understanding of the microorganisms involved would enable improved control of the process.

The main aim of this project was to develop rapid methods and use them to investigate the diversity of methanogenic *Archaea* in landfill. This was to be achieved by the development of molecular biological techniques. In particular, by the amplification of the methanogen-specific *mcrA* gene by PCR, and the subsequent measurement of diversity by analysis of restriction fragment length polymorphisms (RFLP), and by denaturing gradient gel electrophoresis (DGGE). Identification of the individual members of the methanogen communities was to be achieved by the sequencing of *mcrA* genes and phylogenetic analysis. The aim was to compare the methanogen populations in several landfills, and try to identify groups that are ubiquitous in landfill, and therefore possibly important in the degradation process.

An objective of the project was to design oligonucleotide probes suitable for the rapid characterisation of methanogen populations in landfill. This was to be achieved by the sequencing of *mcrA* from described species and from landfill samples. This would provide an expanded database of *mcrA* sequences upon which to base the design of the probes.

A secondary aim of the project was to develop the same molecular biological techniques for investigation of acetogenic *Bacteria* in landfill.

2 MATERIALS AND METHODS

2.1 Chemicals and reagents

All chemicals and reagents were purchased from the following sources, except where specified otherwise:

General chemicals

- BDH Merck
- Sigma Aldrich

Media components

- Difco
- Lab M
- Oxoid

Gases

- Messers

Molecular biology reagents

- Molecular weight markers
  - Amersham Pharmacia Biotech
  - Boehringer Mannheim
  - Gibco BRL LifeTechnologies
- Restriction enzymes
  - Boehringer Mannheim
  - New England Biolabs (NEB)
  - Northumbria Biologicals (NBL)
- dNTPs
  - Boehringer Mannheim
  - Sigma

2.2 Collection of excavated refuse and leachate samples from landfills

Samples of buried waste and/or leachate were collected from five landfill sites. Details of the sites and samples are shown in the results (Table 3.1). Collection of buried waste from the landfill sites was arranged to coincide with the drilling of gas monitoring wells or leachate pumping boreholes. 20 to 50kg of excavated refuse was packed into double-lined black plastic sacks or 25L plastic barrels. The sacks were compressed to remove as much air as possible before being sealed with cable-ties. The barrels were packed tightly to exclude as much air as possible, then sealed. Leachate samples were collected from wells that had been pumping up to a week before sampling to ensure that the leachate was relatively fresh. The leachate was collected in sterile, 25L, plastic containers, which were filled completely to exclude air, transported to the laboratory and stored at 4°C until processed.

2.3 Processing of samples for DNA extraction

2.3.1 Construction and operation of accelerated model landfill reactors

Accelerated model landfill reactors were used to obtain samples of actively growing landfill methanogens. These reactors were designed and had previously been operated at CAMR by P. Riley (personal communication) and Luton (1996). Approximately 10kg of excavated refuse were sorted by hand to remove large pieces of inert material, such as glass, plastic, metal and stones. 7.5kg of this sorted material were packed into accelerated model landfill reactors. The reactors were constructed from plastic barrels (13.8L), with screw-top lids (Solent Plastics). Taps were fitted to the barrels, two in the top, for gas collection and water addition and one in the bottom to collect liquid from the reactor. 2kg of washed and autoclaved gravel was placed in

the bottom of each barrel followed by 7.5kg of landfill material overlaid with a further 2kg of gravel. A water disperser (a plastic lid with a pattern of concentric holes drilled through it) was placed on top of the gravel. The screw-top lids were sealed tight and the reactors flushed with oxygen-free nitrogen for 10 minutes to accelerate the establishment of anaerobic conditions. Gas-bags with a volume of 5L (Stedim) were attached to each reactor to collect the gas produced. 1L of sterile deionised water was added to each reactor and the reactors were incubated at 37°C. The proportion of carbon dioxide, oxygen and methane in the gas produced by each reactor was monitored daily with a LGF20 gas meter (The Advanced Development Company) to check for the onset of methanogenesis. Once the reactors had started producing methane, samples of the liquid that had accumulated at the bottom of the barrels were collected. 50ml of liquid sample from the reactors were centrifuged at 7000×g for 10 minutes at room temperature. The pellet was resuspended in 600µl of sterile pyrogen free water (Parkfields) and the DNA extracted using the RiboLyser phenol/chloroform method (2.4.1).

2.3.2 Processing of excavated refuse samples for direct DNA extraction

Ten 20g sub-samples were taken from the excavated refuse sample, and sorted by hand to remove any hard or sharp particles that might puncture the Stomacher bags. Each sub-sample was double-bagged in Stomacher bags, to reduce the risk of puncturing. 180ml of sterile water was added to each sample and the samples processed for 2 minutes in a Stomacher 400 (Seward). The homogenised samples were pooled and then centrifuged at 300×g for 5 minutes to remove the large particles. The supernatant was centrifuged at 7000×g for 30 minutes to pellet the cells. The pellet was resuspended in 10ml of sterile water and DNA was extracted from 600µl of

this suspension using the RiboLyser phenol/chloroform method (2.4.1). DNA was also extracted directly from 500mg of the pellet using the "Fast DNA Spin Kit for Soil" method (2.4.2).

2.3.3 Processing of leachate samples

2 litres of each leachate sample were centrifuged at 7000×g, 0-4°C for 30 minutes. The pellets were resuspended in 10ml of sterile water and DNA was extracted from 600µl of this suspension using the RiboLyser phenol/chloroform method (2.4.1). DNA was also extracted directly from 500mg of the pellet using the "Fast DNA Spin Kit for Soil" method (2.4.2).

2.4 DNA extraction and purification

2.4.1 RiboLyser phenol/chloroform method

600µl of suspension was added to a RiboLyser™ BLUE tube (Hybaid) containing acid-washed, silica/ceramic matrix. 500µl of phenol equilibrated with TE buffer (pH8.0) (10mM Tris, 1mM EDTA) and 100µl of chloroform isoamyl alcohol (24:1 mixture) were also added to the tubes. The tubes were placed in the RiboLyser™ instrument (Hybaid) and processed for 30 seconds at a speed setting of 6 metres per second. The tubes were placed on ice for 1-2 minutes, followed by centrifuging for 10 minutes at 4°C in a refrigerated microcentrifuge to separate the phases. The top phase was recovered to a microcentrifuge tube leaving approximately 15% of the top phase behind to avoid disturbance of the interphase and contamination of the final sample with protein. 300µl of chloroform isoamyl alcohol was added to the microcentrifuge tube, which was then vortexed for 10 seconds, followed by centrifuging at high speed for 2 minutes to separate the phases. Again the top phase

was removed to a microcentrifuge tube avoiding the interphase material. 500µl of 2-propanol was added to the microcentrifuge tube, the solution was mixed and incubated at –20°C for at least 30 minutes. The solution was centrifuged for 5-10 minutes at high speed to pellet the precipitated DNA. The pelleted DNA was then washed twice with 250µl of ethanol and dried for 15-30 minutes in a vacuum desiccator. The DNA pellet was dissolved in 50-100µl of sterile deionised water and stored at –20°C.

2.4.2 "FastDNA SPIN Kit For Soil" method

The FastDNA SPIN Kit for Soil (Bio 101) was used according to the manufacturer's instructions. 500mg of pelleted material from the leachate or solid samples was added to each MULTIMIX 2 Tissue Matrix Tube (Bio101) containing a mixture of different sized ceramic and silica particles. 978µl of sodium phosphate buffer (Bio101) and 122µl of MT buffer (Bio101) were added to each MULTIMIX tube. The tubes were secured in the RiboLyser instrument and processed for 30 seconds at speed 5.5. The MULTIMIX tubes were centrifuged at 14000×g for 15 minutes and the supernatant was transferred to a clean microcentrifuge tube. 250µl of PPS reagent (Bio101) was added to each tube and the solution mixed by inverting ten times. The tubes were centrifuged at 14000×g for 5 minutes to pellet the precipitate. The supernatant was transferred to a clean microcentrifuge tube and 1ml of Binding Matrix Suspension (Bio101) was added to each tube. The tubes were inverted by hand for 2 minutes to allow binding of DNA to the matrix, then placed in a rack for 3 minutes to allow settling of the silica matrix. 500µl of supernatant was removed from each tube and discarded. The binding matrix was resuspended in the remaining supernatant and the mixture was transferred to a SPIN Filter (Bio101). The SPIN Filters were centrifuged

at 14000×g for 1 minute and the flow-through was discarded. 500µl of SEWS-M (Salt Ethanol Wash, Bio101) was added to each SPIN Filter and the SPIN Filters were centrifuged at 14000×g for 1 minute. The flow-through was discarded and the SPIN Filters were centrifuged at 14000×g for 2 minutes to dry the matrix of residual SEWS-M wash solution. The SPIN Filters were placed in new Catch Tubes and air dried with the caps open for 5 minutes at room temperature. 50µl of DES (DNA Elution Solution; ultra-pure water, Bio101) was added to each SPIN Filter and the silica pellet was resuspended by brief vortexing. The SPIN Filters were centrifuged at 14000×g for 1 minute to transfer the eluted DNA to the Catch Tubes. The DNA solution in the Catch Tubes was stored at –20°C.

2.4.3 Further purification and concentration of DNA extracts and PCR products

For successful PCR and cloning, it was sometimes necessary to further purify the DNA extract prior to PCR and then to clean and concentrate the PCR product prior to cloning. This was accomplished using a Gene Clean II Kit (Bio 101) as described below. PCR products were also Gene Cleaned prior to sequencing. The DNA extract or PCR reaction was transferred to a 1.5ml microcentrifuge tube and 3 volumes of 4M sodium iodide solution (Bio101) added. 10µl of resuspended Glassmilk (Bio 101) was added and the mixture incubated on ice for 1 hour with occasional vortexing. The Glassmilk was pelleted by centrifugation for 1 minute at high speed in a microcentrifuge. The aqueous layer was removed and the pellet resuspended in 700µl of "New Wash" buffered solution (Bio 101). The Glassmilk was repelleted and washed twice more. After the third wash the Glassmilk was repelleted, the wash solution discarded and the pellet vacuum desiccated for 15 minutes. The pellet was

resuspended in 20µl of sterile deionised water, incubated in a 50°C waterbath for 3 minutes, and the Glassmilk pelleted by centrifuging for 3 minutes at 14,000×g in a microcentrifuge. The supernatant containing the purified DNA was recovered.

PCR products were gel purified using a modification of the above procedure. PCR products were run on an agarose gel and stained with ethidium bromide (2.6). The desired band was viewed on an UV transilluminator (312nm) and the band was cut out of the gel with a scalpel. 4.5 volumes of 4M NaI solution and 0.5 volumes of TBE modifier solution (Bio 101) were added to the gel slice in a microcentrifuge tube (1 gel volume = 0.1g). The mixture was incubated at 55°C until the agarose was completely melted, then 10 - 20µl of Glassmilk was added and the procedure above followed.

2.5 PCR amplification of the *mcrA* gene

An approximately 500 base pair (bp) fragment of the *mcrA* gene from methanogens was amplified by the polymerase chain reaction (PCR) using primers designed by Luton (1996). Oligonucleotides, obtained from the Structural Sciences Department, CAMR, were synthesized on a 380B DNA synthesizer (Applied Biosystems). The sequences of the primers are shown below:

mcrA-P1 forward	5'-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3'
mcrA-P3 reverse	5'-TTCATTGCRTAGTTWGGRTAGTT-3'

50µl PCRs were set up in 0.2ml thin wall PCR tubes. The reaction mixture contained: 34µl of sterile deionised water (Parkfields), 5µl of 10× PCR buffer (Boehringer

Mannheim), 5µl of dNTP stock solution (2mM solution of each of the 4 bases; dATP, dTTP, dCTP and dGTP), 1µl of 125µM solution of forward primer, 1µl of 125µM solution of reverse primer, 2µl of template DNA solution and 2µl of 0.5unit μl^{-1} *Taq* DNA polymerase (Boehringer Mannheim). Hot start PCR was used to prevent mis-priming. This involved the addition of the *Taq* DNA polymerase once the temperature of the reaction mixture had reached in excess of 80°C during the first cycle. Thermal cycling was performed in an UNO II Thermocycler (Biometra) or a Programmable Thermal Cycler (MJR). If the MJR machine was used 30µl of light mineral oil (Sigma) was layered over the aqueous solution to prevent evaporation. This was not necessary with the Biometra machine as it had a hot lid, which prevents evaporated liquid condensing in the top of the PCR tube. The cycle parameters used were:

mcrA PCR programme no. 1

95°C for 45 seconds (denaturation),
54°C for 45 seconds (annealing),
ramp to 72°C at 0.1°C sec^{-1},
72°C for 2 minutes (extension),
5 cycles, then
95°C for 45 seconds (denaturation),
54°C for 45 seconds (annealing),
72°C for 2 minutes (extension),
35 cycles, then
72°C for 5 minutes (final extension),
hold at 4°C.

The temperature ramp rate was at maximum (1°C sec^{-1} for the MJR machine and 2°C sec^{-1} for the Biometra) except where specified. For each set of PCR reactions a negative control was included. This contained all the constituents of the reaction mix except template DNA. The production of a PCR product from this control would therefore indicate the presence of contaminating DNA in 1 of the reaction components.

Strong templates, such as when reamplifying PCR products, sometimes generated non-specific amplification products. This could be overcome by adding less template (1µl of PCR product) and altering the cycling parameters to:

mcrA PCR programme no. 2

95°C for 45 seconds (denaturation),
60°C for 45 seconds (annealing),
72°C for 2 minutes (extension),
25 cycles, then
72°C for 5 minutes (final extension),
hold at 4°C.

If the above PCR protocols failed to yield sufficient product from low template concentrations, PCR was performed with an alternative DNA polymerase, Platinum *Pfx* DNA polymerase (Gibco BRL). The method was the same, except 10× *Pfx* Amplification buffer (Gibco BRL) and 50mM $MgSO_4$ solution (Gibco BRL) were used instead of the 10× PCR buffer and an extension temperature of 68°C was used instead of 72°C. Inhibition of the PCR was often overcome by diluting the template DNA. Dilutions between 1 in 2 and 1 in 100 were used.

2.6 Agarose gel electrophoresis

The success of DNA extracts and PCR amplifications were checked by agarose gel electrophoresis. 2µl of gel loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 15% (w/v) Ficoll Type 400) was mixed with 10µl of DNA extract or PCR reaction. 12µl of each sample was loaded onto a mini (10×8×0.5cm) agarose gel, prepared and run in 0.5× TBE buffer (44.5mM Tris-HCl, 44.5mM boric acid, 10mM EDTA, pH8.0). 0.8% (w/v) agarose (Gibco BRL) was used for DNA extracts, and 2% (w/v) agarose for PCR products. A molecular weight marker was

loaded onto each gel. The gel was run at 100V for 1 hour, stained for 10 minutes in 100ml of 0.5× TBE containing 0.25μg ml^{-1} ethidium bromide. The DNA was then visualised on an UV transilluminator (312nm) and a digital record made using 'The Imager' system (Appligene).

2.7 Cloning of PCR products

PCR products were cloned using the Original TA Cloning Kit (Invitrogen). The PCR product was ligated into p*CR*®2.1 vector. The ligation reaction contained: 5μl of sterile water (Invitrogen), 1μl of 10× ligation buffer (Invitrogen), 1μl of fresh PCR product, 2μl of p*CR*®2.1 vector (25ηg μl^{-1}) (Invitrogen) and 1μl of T4 DNA ligase (4.0 Weiss units) (Invitrogen). The ligation reaction was incubated overnight at 14°C.

E. coli INVαF' competent cells (Invitrogen) were transformed with the ligated vector. 2μl of β-mercaptoethanol (Invitrogen) was added to each vial of competent cells and mixed gently. The competent cells were incubated on ice for 30 minutes, heat shocked for 30 seconds at 42°C and placed on ice for 2 minutes. 250μl of SOC medium (2.0% tryptone, 0.5% yeast extract, 10.0mM NaCl, 2.5mM KCl, 10.0mM $MgCl_2 \cdot 6H_2O$, 20.0mM glucose; Invitrogen) was added to each vial and the vials incubated at 37°C for 1 hour at 225rpm in a rotary shaking incubator. The vials of transformed cells were then placed on ice. 50μl and 200μl of transformed cells were spread on LB agar plates (1.0% (w/v) tryptone, 0.5% (w/v) yeast extract, 1.0% (w/v) NaCl, 15g L^{-1} agar, pH7.0) containing 50μg ml^{-1} of ampicillin and 40μg ml^{-1} of X-Gal. The plates were incubated overnight at 37°C. The plates were examined for blue and white colonies. When the cloning worked efficiently there were 50 - 200

colonies per plate and about 80% were white. One hundred white colonies were picked off the plates and streaked onto fresh LB agar + ampicillin plates. These plates were incubated overnight at 37°C. Colonies were picked off these plates and the DNA extracted as described below (2.8).

2.8 Isolation of DNA from transformants

Transformant colonies were resuspended in 50µl of GeneReleaser™ diluent solution (10mM Tris-HCl, 50mM KCl and 0.033% (v/v) Triton X-100, pH8.3). 20µl of resuspended cells were added to 20µl of GeneReleaser™ resin (Cambio) and mixed by vortexing. The tubes containing the cells and GeneReleaser™ resin were sealed, placed in a plastic rack and the rack was placed in a microwave oven. A plastic tray containing 150ml of water was placed on top of the rack to act as a heat sink. The whole assembly was heated in a microwave on full power (650Watts) for 15 minutes. The cells and GeneReleaser™ resin were vortexed again, then centrifuged for 2-3 minutes at high speed in a microcentrifuge to pellet the resin and cell debris. The supernatant was carefully removed to a fresh microcentrifuge tube without disturbing the pellet. The cloned *mcrA* gene fragment was amplified from the supernatant by PCR as described above.

2.9 Restriction analysis

Cloned *mcrA* gene PCR products were analysed by restriction analysis. The PCR products generated by amplification of the cloned *mcrA* genes were digested with a single restriction endonuclease *Taq*I (cut site: T/CGA) or *Rsa*I (cut site: GT/AC). 18µl of PCR product was incubated with 2µl (20units) of *Taq*I at 65°C or *Rsa*I at 37°C for 1 hour. 4µl of gel loading buffer was mixed with the digest prior to loading

onto a large (25×20×0.5cm) 4% (w/v) agarose gel, prepared and run in 0.5× TBE buffer. A 50bp DNA ladder was also loaded onto the gel as a molecular weight marker. The gel was run at 90 volts for 5 hours, stained for 30 minutes in 400 ml of 0.5× TBE containing 0.25μg ml^{-1} ethidium bromide, then destained in 0.5× TBE for 30 minutes. The DNA was visualised on an UV transilluminator (312nm) and a digital record made using 'The Imager' system (Appligene).

2.10 Sequencing of PCR products

Automated sequencing of *mcrA* gene PCR products was performed by the Structural Sciences Department at CAMR using an ABI 377 sequencer (Applied Biosystems). The machine performs cycle sequencing using a thermostable polymerase (Ampli*Taq*, Perkin Elmer) and a fluorescent dye chain terminating mix (Big Dye™, Perkin Elmer). PCR products were generated as above (2.5), but using primers that incorporated the M13 universal forward and reverse primer binding sites into the PCR product. This enabled the M13 universal primers to be used in the sequencing reaction. The sequences of the *mcrA*/M13 primers are shown below:

mcrA/M13 forward

5'-TGTAAAACGACGGCCAGTGGTGGTGTMGGATTCACACARTAYGCWAC AGC-3'

mcrA/M13 reverse

5'-CAGGAAACAGCTATGACC TTCATTGCRTAGTTWGGRTAGTT-3'

The reaction volume for the PCR was 100μl. Each reaction contained: 74μl of sterile

deionised water (Parkfields), 10µl of 10× PCR buffer (Boehringer Mannheim), 10µl of dNTP stock solution (2mM solution of each of the 4 bases; dATP, dTTP, dCTP and dGTP), 1µl of 125µM solution of forward primer, 1µl of 125µM solution of reverse primer, 2µl of template DNA and 2µl of 1unit μl^{-1} *Taq* DNA polymerase (Boehringer Mannheim). All the other conditions were the same as described above. 10µl of each PCR was run on a 2% agarose mini-gel to check for product. The remaining 90µl was purified and concentrated using the Gene Clean II kit as described above (2.4.3). The purified DNA was sequenced.

2.11 Sequencing of the *mcrA* gene from methanogen pure cultures

2.11.1 Growth of methanogens from pure cultures

Pure cultures of methanogens were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Table 2.1), and grown in the media recommended by the DSMZ. Each medium was prepared as recommended by the DSMZ, except media 119, 279 and 321 from which sludge fluid and rumen fluid were omitted. 100ml of medium were dispensed into 160ml Wheaton bottles, which were sealed with rubber septa and aluminium crimp caps (Chromatography Services, Liverpool). The media was inoculated with 1ml of pure culture (10ml for *Methanosaeta concilii*) by injecting through the rubber septa with sterile needles and syringes. The cultures were incubated at the temperature recommended by the DSMZ for 1 to 4 weeks.

Table 2.1 Cultures of methanogens obtained from the DSMZ

Organism	DSMZ accession no.	DSMZ medium
Methanobacterium bryantii	863	119[a]
Methanobacterium espanolae	5982	506
Methanobacterium formicicum	1312	119[a]
Methanobrevibacter arboriphilicus	1125	119[a]
Methanobrevibacter ruminantium	1093	119[a]
Methanocorpusculum aggregans	3027	321[a]
Methanocorpusculum bavaricum	4179	279[a]
Methanocorpusculum parvum	3823	279[a]
Methanoculleus bourgensis	3045	332
Methanohalophilus halophilus	3094	329
Methanopyrus kandleri	6324	511
Methanosaeta concilii	3671	334
Methanosarcina mazei	2053	120
Methanospirillum hungatei	864	119[a]

[a] Sludge fluid and rumen fluid were omitted from these media.

2.11.2 Monitoring growth of methanogens

Growth of the cultures was monitored by visual inspection of the opacity of the medium and by detecting methane in the headspace gas. 0.5ml samples of headspace gas were injected into a gas chromatograph (Pye Unicam series 104 GC fitted with a 4mm internal diameter glass column containing silica gel packing at 100°C with nitrogen carrier gas and a flame ionisation detector at 250°C). Output was obtained from a HP 3390A integrator (Hewlett Packard). Growth of methanogens was determined by the presence of methane. Methane was purged from the cultures after each measurement by flushing the headspace gas with the appropriate gas mix for that medium.

2.11.3 Extraction of DNA from pure cultures of methanogens

DNA was extracted from pure cultures of methanogens using the GeneReleaser method (2.8). 1ml of culture was centrifuged at 10,000×g for 10 minutes at 4°C. The supernatant was removed and the pellet was resuspended in 20µl of GeneReleaser diluent solution. 20µl of GeneReleaser resin was added to the suspended pellet and the GeneReleaser protocol was followed as described in section 2.8.

2.11.4 Amplification and sequencing of the *mcrA* gene from pure cultures

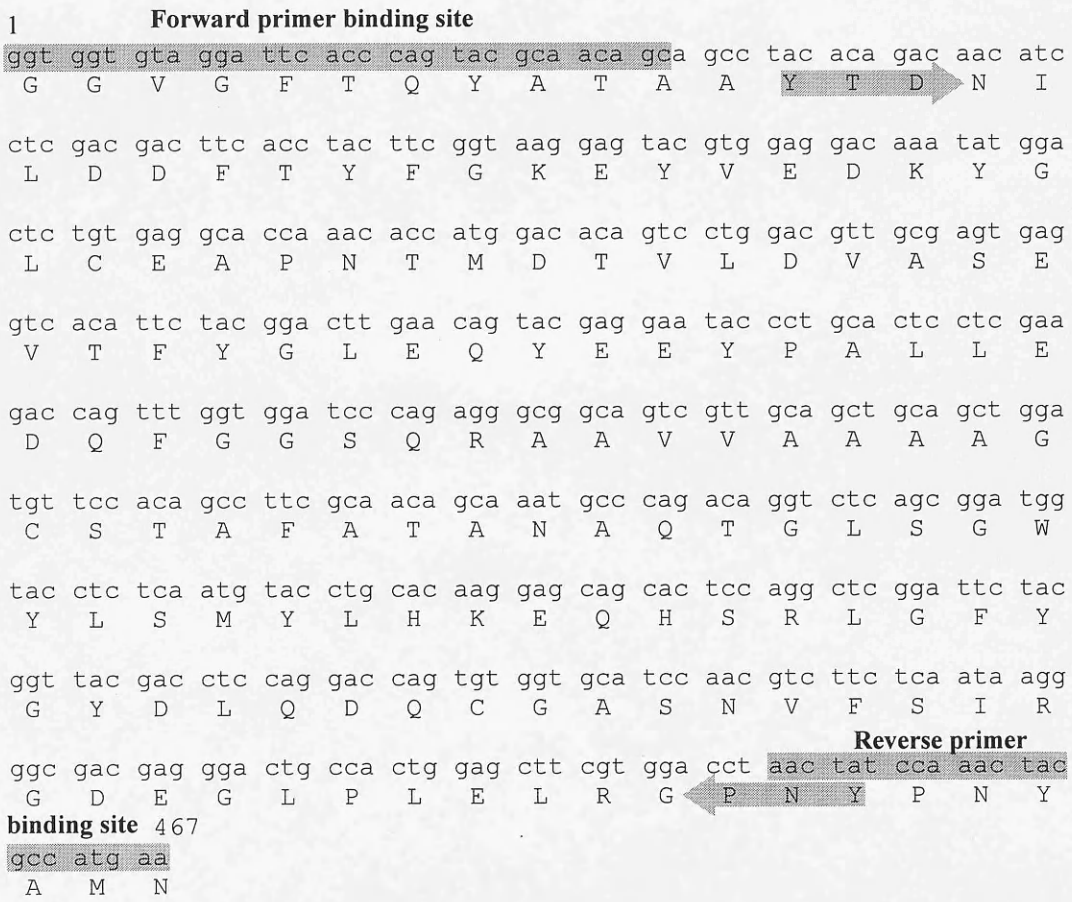
A 464-491bp fragment of the *mcrA* gene was amplified by PCR from the DNA extracted from pure cultures of methanogens using the method described in section 2.5 and 2.10. The sense and anti-sense strands of the PCR products were sequenced as described in section 2.10.

2.12 Phylogenetic analyses

McrA DNA sequences were translated into amino acid sequences using EDITSEQ (DNAStar). The peptide sequences were edited so that they all corresponded to the region of methyl coenzyme M reductase subunit A shown in Figure 2.1. The lengths of the peptide sequences were from 137 to 146 amino acids. The peptide sequences were aligned using PILEUP in the GCG suite of programmes (Wisconsin Package Version 10.1, Genetics Computer Group), which was accessed through SEQWEB Version 1.2 (Genetics Computer Group).

The sequence data was subjected to phylogenetic analysis using programmes in PHYLIP (Phylogeny Inference Package) Version 3.57c (Felsenstein, 1995). Distance matrices were calculated from the peptide sequences using the Dayhoff

Figure 2.1 Positions of *mcrA* PCR primers and amino acid sequence used for phylogenetic analyses.



Legend: The figure shows the partial DNA and corresponding amino acid sequence of *mcrA* from *Methanothermobacter thermoautotrophicus* strain delta H, GenBank accession number U10036. The binding sites of the forward and reverse PCR primers are indicated by the boxes. The start and end of the region of the amino acid sequence used for phylogenetic analysis is indicated by the block arrows.

PAM matrix method in the programme PROTDIST (PHYLIP 3.57c). Phylogenetic trees were constructed from the calculated distance values using two programmes, FITCH (PHYLIP 3.57c), which employs the method of Fitch & Margoliash (1967), and NEIGHBOR (PHYLIP 3.57c), which employs the Neighbor-Joining method of Saitou & Nei (1987). Bootstrap resampling analysis with 100 replicates was performed using the programme SEQBOOT (PHYLIP 3.57c), to estimate the confidence of tree topologies. Consensus trees were determined using CONSENSE (PHYLIP 3.57c) and the trees were displayed using TREEVIEW Version 1.6.1 (Page, 1996). The programme PROTPARS (PHYLIP 3.57c) was used in place of the programmes PROTDIST, FITCH and NEIGHBOR to construct phylogenetic trees from the same data sets. PROTPARS uses the parsimony method of Felsenstein (1988). Bootstrap resampling analysis was performed as described above.

The cloned *mcrA* sequences were investigated for the presence of chimeric sequences. The nucleotide sequences of 90 cloned *mcrA* PCR products from landfill and 19 sequences from described methanogen species were aligned using PILEUP (Gap creation penalty 1, gap extension penalty 1). Phylogenetic trees were constructed based on the whole sequence (440 bases) and using the 5' or 3' 200 bases of sequence. Distance values were calculated using the programme DNADIST (PHYLIP 3.57c) (Kimura 2-parameter method), and phylogenetic trees were constructed using NEIGHBOR (Neighbor-Joining method). The trees were compared by eye, looking for taxa that changed position radically between trees, i.e. appeared to group with different, clearly distinct clusters.

2.13 Probing for the *mcrA* gene

2.13.1 3'-End labeling oligonucleotides with Digoxigenin-11-ddUTP

Oligonucleotide probes for the *mcrA* gene were synthesized by Structural Sciences at CAMR and labeled with digoxigenin-11-ddUTP using the DIG Oligonucleotide 3'-End Labeling Kit (Roche Molecular Biochemicals) according to the manufacturer's instructions.

For each reaction the following reagents were added to a sterile microcentrifuge tube (on ice):

Reagents	**Volume**	**Final concentration**
5× reaction buffer	4µl	1×
$CoCl_2$ solution	4µl	5mM
Oligonucleotide	100pmol	5pmol μl^{-1}
DIG-ddUTP	1µl	0.05mM
Terminal Transferase	1µl	2.5 units μl^{-1}
Sterile, distilled water	to 20µl	-
Total volume	20µl	

The reactions were incubated at 37°C for 15 minutes, then placed on ice. 1µl of EDTA (200mM, pH8.0) was added to each tube to terminate the labelling reaction.

2.13.2 Dot blotting

The PCR products from the cloned *mcrA* fragments from each of the solid and leachate samples were dot blotted onto nylon membrane. PCR products were denatured by heating at 95°C for 10 minutes, then placing immediately on ice. 1µl of each PCR product was spotted onto a positively charged nylon membrane (Roche Molecular Biochemicals). Up to 87 PCR products of clones from each landfill sample

were spotted onto nylon membranes. Eight PCR products were selected as positive controls for each of the probes and spotted onto each of the membranes. The DNA was fixed onto the membranes by baking at 120°C for 30 minutes.

2.13.3 Hybridisation

The dot blots were prehybridised in 30ml of prehybridisation solution (5×SSC, 0.1% (w/v) N-lauroyl-sarcosine, 0.02% (w/v) SDS, 2% Blocking Reagent; Roche Molecular Biochemicals) at the melting temperature (Tm) of the probe for 2 hours in a Hybridiser HB-1 (Techne). The Tm was calculated using the formula: Tm(°C) = 4(G+C)+2(A+T). The prehybridisation solution was removed and 6ml of hybridisation solution (prehybridisation solution containing 1pmol ml^{-1} DIG-labeled oligonucleotide) added to the roller tubes. Hybridisation was carried out for 30 minutes at the Tm of the probe. The membrane was then washed twice for 5 minutes in 50-100ml of 2× Wash solution (2×SSC, 0.1% SDS), twice for 15 minutes in 50-100ml of 0.5× Wash solution (0.5×SSC, 0.1% SDS), and twice for 15 minutes in 50-100ml of 0.1× Wash solution (0.1×SSC, 0.1% SDS), at room temperature in sterile plastic trays with vigorous agitation. (Note: 1×SSC contains 0.15M NaCl, 15mM sodium citrate, pH7.0.)

2.13.4 Chemiluminescent detection

The hybridised probe was detected by chemiluminescent detection. This was carried out using the DIG Chemiluminescent Detection Kit (Roche Molecular Biochemicals) according to the manufacturer's instructions.

The hybridised membrane was placed in a sterile, plastic tray and equilibrated for 1

minute in washing buffer (100mM maleic acid, 150mM NaCl; pH7.5; 0.3% (v/v) Tween 20) with gentle agitation. The membrane was blocked by gently agitating it for 60 minutes in blocking solution (1% (w/v) Blocking reagent dissolved in 100mM maleic acid, 150mM NaCl; pH7.5). Anti-Digoxigenin-AP (750units ml^{-1} Anti-Digoxigenin, Fab fragments conjugated to alkaline phosphatase, Roche Molecular Biochemicals) was diluted 1:10,000 in blocking solution. The blocking solution was poured off and the membrane was incubated for 30 minutes in the antibody solution with gentle agitation. The membrane was transferred to a new sterile, plastic tray and washed twice, 15 minutes per wash, in washing buffer with gentle agitation. The washing buffer was poured off and the membrane equilibrated for 2 minutes in detection buffer (100mM Tris-HCl, 1mM EDTA; pH8.0). CSPD (25mM disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.$1^{3,7}$]decan}-4-yl)phenyl phosphate, Roche Molecular Biochemicals) was diluted 1:100 in detection buffer. 15ml of diluted CSPD was pipetted into a sterile, plastic tray and the membrane was placed in the tray using sterile forceps. The tray was tilted until the membrane was thoroughly saturated and the membrane was incubated at room temperature for 5 minutes. The membrane was removed from the CSPD solution and any excess liquid allowed to drip off. The CSPD solution was used for more than one membrane. The membrane was wrapped in cling film to prevent it from drying out and incubated at 37°C for 15 minutes to allow the alkaline phosphatase chemiluminescent reaction to reach a steady state.

2.13.5 Detection of chemiluminescent signal

The chemiluminescent signal was detected by exposing X-ray film (Fuji Medical) to the membrane for 8-16 hours. The X-ray film was developed using Kodak GBX

Developer and Fixer solutions in a SS2 Processor (*plh* Medical) and using the method recommended by Kodak.

After chemiluminescent detection the membrane was placed in an X-ray exposure cassette with a sheet of X-ray film and exposed overnight. The X-ray film was then immersed in GBX Developer solution (Kodak) for 5 minutes at 20°C. The film was removed from the developer solution and any excess liquid allowed to drip off. It was then immersed in Stop solution (2.5% (v/v) acetic acid) for 30 seconds at 20°C with agitation. The film was next immersed in GBX Fixer solution (Kodak) for 5 minutes at 20°C with agitation, followed by rinsing in water for 5 minutes at 20°C. The film was air-dried. (Note: Film exposure and development were carried out in complete darkness.)

2.13.6 Stripping of the membranes for reprobing

The membrane was removed from the cling film and washed in sterile, distilled water twice, 1 minute per wash, with vigorous agitation in a sterile, plastic tray. The membrane was incubated twice for 10 minutes at 37°C in alkaline probe-stripping solution (0.2M NaOH, 0.1% (w/v) SDS) with agitation. The membrane was then rinsed twice for 2 minutes in 2× SSC buffer (300mM NaCl, 30mM sodium citrate) with agitation.

2.14 Denaturing gradient gel electrophoresis

DGGE was performed on a DCode system (BioRad). Denaturing polyacrylamide gels (16×20×0.1cm) were prepared with 6% (w/v) acrylamide/bis-acrylamide (37:1 ratio), 2% (v/v) glycerol, 0.1% (v/v) TEMED and 0.03% (w/v) ammonium persulphate in

1.25× TAE buffer (50mM Tris-HCl, 25mM acetic acid glacial, 1.25mM EDTA, pH8.0). 100% denaturing conditions were obtained by adding 40% (v/v) deionised formamide, 42% (w/v) urea to the gel solution. Denaturing gradient gels were formed using the Model 475 Gradient Delivery System (BioRad). Gels were run in 1.25× TAE buffer at constant voltages ranging from 4.5 to 9V cm^{-1} and at different temperatures ranging from room temperature to 70°C.

Gels were run with the following configurations:

- Perpendicular DGGE - the denaturing gradient was perpendicular to the direction of electrophoresis,
- Parallel DGGE - the denaturing gradient was parallel to the direction of electrophoresis,
- CDGE - a constant denaturant concentration,
- TTGE - a temporal temperature gradient was combined with a constant denaturant concentration.

The gels were stained with ethidium bromide or SYBR Green I (Molecular Probes), by immersing for 30 minutes in 1.25× TAE containing either 0.25μgml^{-1} ethidium bromide or a 1 in 10,000 dilution of SYBR Green I stock solution. The gels were stained in complete darkness, as SYBR Green I deteriorates rapidly when exposed to light. The stained gels were viewed on an UV transilluminator (312nm).

A GC-clamp was attached to PCR products used for DGGE by adding a 40bp GC-rich sequence to the 5' end of the *mcrA* reverse primer. The GC-clamp/*mcrA* reverse primer was used with the *mcrA* forward primer to amplify *mcrA* gene fragments by PCR as described in section 2.5. The sequence of the GC-clamp was obtained from Sheffield *et al* (1989).

GC-clamp/*mcrA* reverse primer

5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG

TTCATTGCRTAGTTWGGRTAGTT-3'

2.15 Isolation and identification of novel methanogen species from landfill

140 anaerobic culture tubes containing 10ml of a *Methanobacterium* medium (see below) were inoculated with 10^{-10} to 10^{-12} dilutions of leachate from the model landfill reactor containing waste from the Odcombe landfill site. The cultures were incubated at 37°C, growth of methanogens was checked for by detecting methane in the head space gas using gas chromatography (2.11.2). DNA was extracted from the cultures using GeneReleaser and the *mcrA* gene was amplified by PCR as described above (2.5). PCR products were analysed by restriction analysis, as described above (2.9), to identify cultures containing single methanogen species.

Seven primers for the 16S rRNA gene were tried in different combinations to amplify fragments of the 16S rDNA from the methanogen containing cultures (Table 2.2). Two primer pairs, 0348a Forward plus 1100a Reverse and 0802 Forward plus 1525 Reverse, were used to amplify partially overlapping 750bp fragments of the 16S rDNA from the cultures identified by restriction analysis. These PCR products were sequenced after amplification with the M13/16S rDNA primers (Table 2.2). Strongest matches to sequences in the GenBank database (Benson *et al*, 2000) were identified using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*, 1990).

16S rDNA PCR programme:

95°C for 2 minutes (denaturation),
40°C for 30 seconds (annealing),
ramp to 72°C at 0.1°C sec^{-1},
72°C for 1 minute (extension),
1 cycle, then
95°C for 30 seconds (denaturation),
40°C for 30 seconds (annealing),
ramp to 72°C at 0.1°C sec^{-1},
72°C for 1 minute (extension),
5 cycles, then
95°C for 30 seconds (denaturation),
40°C for 30 seconds (annealing),
72°C for 1 minute (extension),
35 cycles, then
72°C for 6 minutes (final extension),
hold at 4°C.

Table 2.2 16S rDNA primers for PCR and sequencing.

Primer name	*E. coli* numbers	Sequence (5'-3')	*Archaea*	*Bacteria*
0025e Forward[a]	9-25	CTGGTTGATCCTGCCAG	++	
0348a Forward[a]	333-348	TCCAGGCCCTACGGG	++	
0802 Forward[a]	787-802	ATTAGATACCCTGGTA	+	+(taxa specific)
1068 Forward[a]	1053-1068	GCATGGCYGYCGTCAG	++	+
0498ea Reverse[a]	511-498	CTTGCCCRGCCCTT	For Euryarchaeota	
1100a Reverse[a]	1115-1100	TGGGTCTCGCTCGTTG	++	
1525 Reverse[a]	1541-1525	AAGGAGGTGATCCAGCC	++	++
M13/0348a Forward	-	TGTAAAACGACGGCCAGTTCCAGGCCCTACGGG	Primers for sequencing	
M13/0802 Forward	-	TGTAAAACGACGGCCAGTATTAGATACCCTGGTA		
M13/1100a Reverse	-	CAGGAAACAGCTATGACCTGGGTCTCGCTCGTTG		
M13/1525 Reverse	-	CAGGAAACAGCTATGACCAAGGAGGTGATCCAGCC		

[a] Primer sequences obtained from Achenbach & Woese (1995).
++ = Very good.
+ = Fair to good.

***Methanobacterium* medium** (medium 119, DSMZ)

KH_2PO_4	0.5	g L^{-1}
$MgSO_4 \cdot 7H_2O$	0.4	g L^{-1}
NaCl	0.4	g L^{-1}
NH_4Cl	0.4	g L^{-1}
$CaCl_2 \cdot 2H_2O$	0.05	g L^{-1}
$FeSO_4 \cdot 7H_2O$	0.002	g L^{-1}
Yeast extract	1.0	g L^{-1}
Sodium acetate	1.0	g L^{-1}
Sodium formate	2.0	g L^{-1}
Sludge fluid (omitted)	50.0	ml
$NaHCO_3$	4.0	g L^{-1}
Resazurin	1.0	mg L^{-1}
DL-Dithiothreitol	0.5	g L^{-1}
Trace elements solution SL-10 (see below)	1.0	ml
Valeric acid	0.5	ml
Isovaleric acid	0.5	ml
α-Methylbutyric acid	0.5	ml
Iosbutyric acid	0.5	ml
Distilled water	940.0	ml

The pH was adjusted to 6.7 – 7.0. The medium was distributed to anaerobic culture bottles, which were sealed, gassed with N_2 and autoclaved. The sterile medium was gassed with 80% H_2 + 20% CO_2 and the culture bottles were pressurised to 2 bar overpressure with 80% H_2 + 20% CO_2.

Trace elements solution SL-10:

HCl (25%; 7.7 M)	10.0	ml
$FeCl_2 \cdot 4H_2O$	1.5	g
$ZnCl_2$	0.07	g
$MnCl_2 \cdot 4H_2O$	0.10	g
H_3BO_3	0.006	g
$CoCl_2 \cdot 6H_2O$	0.19	g
$CuCl_2 \cdot 2H_2O$	0.002	g
$NiCl_2 \cdot 6H_2O$	0.036	g
$Na_2MoO_4 \cdot 2H_2O$	0.024	g
Distilled water	990.0	ml

The $FeCl_2$ was first dissolved in the HCl, then diluted in water. The other salts were then added and dissolved. Finally the solution was made up to 1000.0 ml.

2.16 Growth of homoacetogenic bacteria

Pure cultures of homoacetogenic bacteria were obtained from the DSMZ (Table 2.3), and grown in the media recommended by the DSMZ. Each medium was prepared as recommended by the DSMZ. 100ml of medium were dispensed into 160ml Wheaton bottles, which were sealed with rubber septa and aluminium crimp caps (Chromatography Services). The freeze-dried cultures were rehydrated with 1ml of the appropriate medium. The rehydrated culture was used to inoculate a bottle of media by injecting through the rubber septa with sterile needles and syringes. The cultures were incubated at 37°C (30°C for *C. aceticum* and *A. wieringae*) until visible growth had occurred (1 day - 2 weeks). Growth of the cultures was monitored by visual inspection of the opacity of the medium.

Table 2.3 Cultures of homoacetogenic bacteria obtained from the DSMZ

Organism	DSMZ accession no.	DSMZ medium
Acetobacterium wieringae	1911	135
Clostridium aceticum	1496	135
Moorella thermoacetica	521	60
Ruminococcus productus	3507	339
Sporomusa acidovorans	3132	311

2.17 DNA extraction and purification from homoacetogen cultures

1 - 10ml of culture was centrifuged at 10,000×g for 10 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in sterile deionised water (0.1 – 1ml). The resuspended pellet (100µl) was added to a MULTIMIX 2 Tissue Matrix Tube and the FastDNA SPIN Kit method (2.4.2) was followed to extract and purify the DNA.

2.18 PCR amplification of the FTHFS gene

The formyltetrahydrofolate synthetase (FTHFS) gene was amplified with a range of PCR primers (Table 2.4). The PCR reagents were the same as for the *mcrA* gene (2.5). The cycling parameters were optimised for each pair of primers. The basic PCR cycle parameters were:

FTHFS PCR programme

95°C for 45secs (denaturation),
Tm-5°C for 45secs (annealing),
72°C for 2mins (extension),
30-40 cycles, then
72°C for 7mins (final extension),
4°C hold.

2.18.1 Optimisation of the PCR

To improve the specificity and yield of the PCR with each pair of primers the following alterations were made to the cycle parameters:

- Touchdown PCR;
- Increased the extension time to 3 minutes for products longer than 1kb.

Table 2.4 Primers for PCR amplification of the FTHFS gene

Primer name[a]	Tm (°C)	Sequence (5'-3')
100f26	68	GARCTWTATGGTAARTATAAGGCTAA
109f17	42	GGWAARTAYAARGCYAA
186f21	60	AACAGCTATTAACCCAACTCC
328f17	64	GGTGCCGCCGGCGGTGG
328f17b	58	GGWGCWGCWGGYGGTGG
334f23	70	GCTGGTGGTGGTTATGCTCAAGT
829f20	54	AATATCGCACATGGTTGTAA
820r21	62	ATGTGCGATATTGGCGAATGG
823r17	46	TGWGCRATRTTKGCGAA
992r18	64	GGGCGCGGACGGTAGCCA
997r23	66	TGCATCTTNAGGGCTCKAACAGT
1465r23	62	AATGAGTATTGTGTCTTAGCCAT
1465r17	44	TATTGDGTYTTRGCCAT
1597r22	64	TTGGMAGTCCTGGCATKGTCAT
14f19[b]	54	CCAGTGATATTGAGATTGC
1400r18[b]	56	GTATAGTTGACGCCGTCG

Key: [a] Primer names are based on the *M. thermoacetica* FTHFS gene sequence (GenBank Accession number J02911) with the nucleotide numbering beginning from the start codon.
[b] Used to amplify a 1.387kb fragment of the FTHFS gene from *M. thermoacetica* for use as a DNA probe for homoacetogens.

2.19 Probing for the FTHFS gene

2.19.1 Digoxigenin labelling of FTHFS gene PCR product

The FTHFS gene was amplified from *M. thermoacetica* with primers 14f19 and 1400r18. The PCR product was used as the template in a PCR with the PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals), which enables the incorporation of DIG-dUTP into PCR products during PCR. The kit was used according to the manufacturer's instructions. The thermal cycling parameters were:

FTHFS probe PCR programme

94°C for 60secs (denaturation),
59°C for 60secs (annealing),
72°C for 60secs (extension),
30 cycles, then
72°C for 10mins (final extension),
4°C hold.

The resultant PCR product was gel purified as described in section 2.4.3 and used as a probe for the FTHFS gene.

2.19.2 Southern blotting of FTHFS gene PCR products

PCR products were run on a 0.8% (w/v) agarose mini-gel (2.6). The gel was submerged in denaturation solution (0.5M NaOH, 1.5M NaCl) twice for 15 minutes at room temperature with gentle shaking. The gel was rinsed with sterile, distilled water, then submerged in neutralisation solution (0.5M Tris-HCl, pH7.5; 3M NaCl) twice for 15 minutes at room temperature with gentle shaking. The PCR products were vacuum-blotted onto a positively charged nylon membrane (Roche Molecular

Biochemicals) for 2 hours using 20×SSC (3M NaCl, 300mM Na citrate, pH7.0). The PCR products were fixed to the membrane by baking at 120°C for 30 minutes.

2.19.3 Hybridisation of FTHFS gene probe

The Southern blots were prehybridised in 30ml of prehybridisation solution (5×SSC, 0.1% (w/v) N-lauroyl-sarcosine, 0.02% (w/v) SDS, 2% Blocking Reagent) at 68°C for 2 hours in a Hybridiser HB-1 (Techne). The prehybridisation solution was poured off and 6ml of hybridisation solution (prehybridisation solution containing 2µl of the gel-purified, DIG-labelled PCR product) were added to the roller tubes. The hybridisation was overnight (~16 hours) at 68°C. The membrane was then washed twice for 5 minutes in 50-100ml of 2× Wash solution (2×SSC, 0.1% SDS) at room temperature, twice for 15 minutes in 50-100ml of 0.5× Wash solution (0.5×SSC, 0.1% SDS) at 68°C, then twice for 15 minutes in 50-100ml of 0.1× Wash solution (0.1×SSC, 0.1% SDS) at 68°C in sterile plastic trays.

2.19.4 Chemiluminescent detection

The hybridised probe was detected by chemiluminescent detection as described in section 2.13.4.

2.19.5 Detection of chemiluminescent signal

The chemiluminescent signal was detected by exposing X-ray film (Fuji Medical) to the membrane for 30 minutes to 16 hours. The X-ray film was developed as described in section 2.13.5.

3 Determining methanogen diversity in landfill by PCR-RFLP and DGGE

3.1 INTRODUCTION

The methanogenic *Archaea* play an essential role in the biological decomposition of waste in landfills. They are responsible for the final step in the decomposition process, the conversion of H_2, CO_2, acetate and a few other simple carbon compounds to methane. Knowledge of the diversity and composition of the methanogen communities in landfills would aid our understanding of the decomposition process (Archer, 1989; Lawson, 1989b). As described previously (section 1.3.5), methanogens are a diverse group of obligate anaerobic *Archaea*. Due to their slow growth rate and fastidious nature, the culture of methanogens is difficult and time-consuming. In addition, it is highly likely that methanogen species present in the environment are not culturable using media and growth conditions based on the requirements of described species. Molecular methods such as those utilised in this study are not reliant on culturing the organisms under investigation and therefore avoid the limitations of culture-based methods.

Luton (1996) demonstrated the specific detection of methanogens in environmental samples by amplification of the *mcrA* gene using the polymerase chain reaction (PCR). The *mcrA* gene encodes a sub-unit of the enzyme, methyl coenzyme-M reductase. This enzyme performs the terminal reaction in the methanogenesis pathway (Figure 1.7, section 1.3.5.1) and is believed to be unique to methanogens

(Weiss & Thauer, 1993). Thus, the presence in an organism of this enzyme, and the genes encoding it, would indicate that the organism was a methanogen. In this study, the *mcrA* specific PCR primers designed by Luton (1996) were used to amplify a 464-491bp fragment from DNA extracted from samples of excavated refuse and leachate from landfills.

To determine the diversity and structure of the methanogen community in landfill, the *mcrA* PCR products were cloned and the clone libraries screened for restriction fragment length polymorphisms (RFLP). This PCR-RFLP technique has been used to study microbial diversity in a variety of environments (Braker *et al*, 2000; Costello & Lidstrom, 1999; Weidner *et al*, 1996; Whitby *et al*, 1999). For example, Moyer *et al* (1994) used PCR-RFLP to estimate microbial diversity and community structure around a hydrothermal vent system. Most studies utilising the PCR-RFLP method have used 16S rDNA as the target molecule. Some studies have used other functional genes, for example, Darrasse *et al* (1994) used the *pel* gene to identify strains of *Erwinia carotovora*. Braker *et al* (2000) used PCR-RFLP of the *nirK* and *nirS* genes to investigate the diversity of denitrifying bacteria in marine sediments. This study is the first to use PCR-RFLP analysis of the *mcrA* gene, in the landfill environment.

Denaturing gradient gel electrophoresis (DGGE) was developed as an alternative to the PCR-RFLP method. As described in Table 1.7, DGGE and the related method, temperature gradient gel electrophoresis (TGGE), separate DNA molecules of the same size by differences in the nucleotide sequence. The pattern of bands generated by DGGE/TGGE may be used as a measure of community diversity, and differences in community structure between environmental samples may be identified by

comparing banding patterns from different samples. DGGE/TGGE analysis may be performed directly on PCR products amplified from environmental samples without cloning, and it is therefore a more rapid method compared to PCR-RFLP. Previous studies have used DGGE to measure microbial diversity in a variety of environments including MSW (Ovreas *et al*, 1997; Santegoeds *et al*, 1996; Silvey and Blackall, 1995).

This chapter describes the application of PCR-RFLP to determination of the diversity and structure of methanogen communities in landfill samples. The development of DGGE/TGGE as an alternative to the PCR-RFLP method is also described.

3.2 RESULTS

3.2.1 Landfill samples: processing and DNA extraction

Samples were obtained from five sites around England (Table 3.1).

Table 3.1 Landfill sites and samples.

Landfill site	Type of sample
Cory Environmental Waste Management site, Mucking, Essex	Excavated refuse
Wyvern Waste Management site, Odcombe, Somerset	Excavated refuse
Shanks and McEwan site, Brogborough, Bedfordshire	Two excavated refuse samples from 3m and 18m depth
Hales Waste site, Poyle, Berkshire	Leachate
Hanson Waste Management site, Hermitage, Berkshire	Leachate and excavated refuse samples

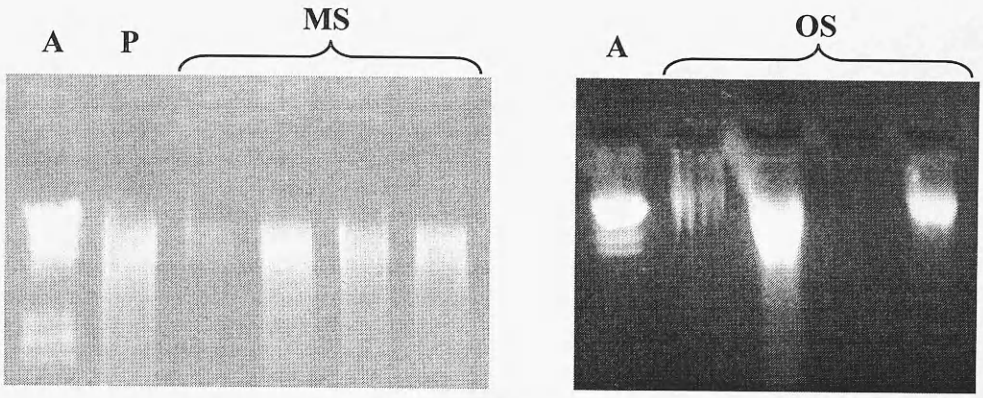
The samples of excavated refuse from the Mucking, Odcombe and Brogborough landfills were used to set up accelerated model landfill reactors, as described in section 2.3.1. All of these reactors produced methane after several days incubation at 37°C. Samples of leachate were obtained from each of the reactors. DNA was extracted directly from the reactor leachate samples from the Odcombe and Brogborough reactors (sections 2.3.1, 2.4). The leachate from the Mucking reactor was used to inoculate an anaerobic culture containing a *Methanobacterium* medium (section 2.15). After two weeks incubation at 37°C, DNA was extracted from this culture (section 2.4).

DNA was extracted directly from the landfill leachate samples from the Poyle and Hermitage landfills (sections 2.3.3, 2.4). DNA was also extracted directly from the excavated refuse sample from the Hermitage landfill (sections 2.3.2, 2.4). DNA recovery, purity and yield were assessed by agarose gel electrophoresis (section 2.6) (Plate 3.1).

3.2.2 PCR amplification and cloning of *mcrA* gene fragments

A ~0.5kb fragment of the *mcrA* gene was amplified by PCR from DNA extracted from the landfill samples using the *mcrA*-specific primers. The PCR amplifications were performed as described in section 2.5, and the PCR products obtained were analysed by agarose gel electrophoresis (section 2.6). Products of the expected size were obtained from all of the landfill samples (Plate 3.2).

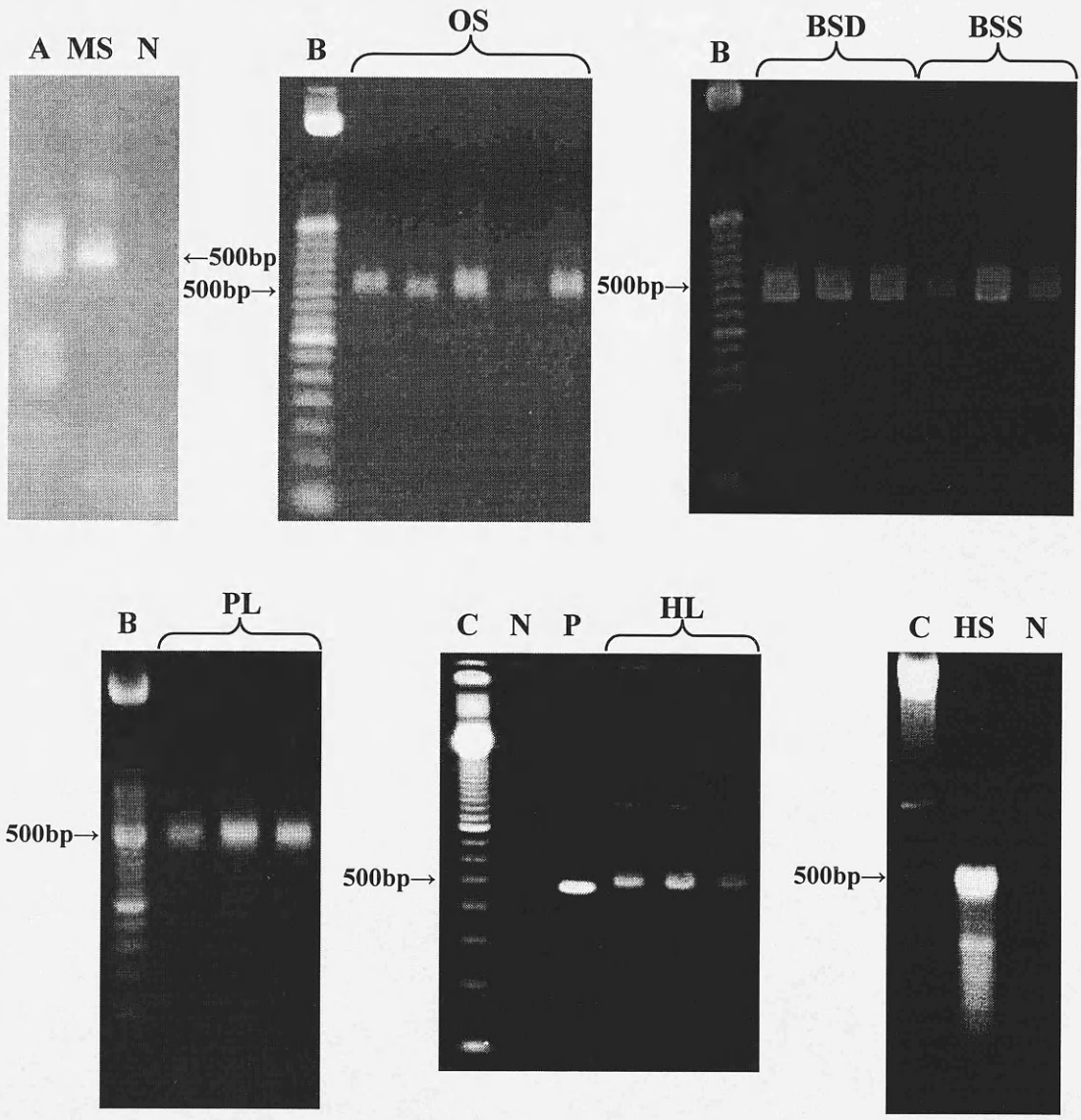
Plate 3.1 DNA extracts from model landfill reactor leachate samples.



Legend: DNA was purified using the RiboLyser phenol/chloroform method (section 2.4.1) from leachate samples taken from the accelerated model landfill reactors containing waste from the Mucking and Odcombe landfills. Samples of DNA (10µl) were run on a 0.8% agarose gel. The DNA was stained with ethidium bromide and visualised at 312nm.

Lanes: A - *Hind* III λ DNA;
P - positive control (*Bacillus stearothermophilus* DNA);
MS - Mucking;
OS - Odcombe

Plate 3.2 PCR amplification of a fragment of the *mcrA* gene from landfill DNA extracts.



Legend: Products from the PCR amplification of the *mcrA* gene were run on a 2% agarose gel, stained with ethidium bromide and visualised at 312nm.

Lanes: A - Molecular weight marker V (Boehringer Mannheim);
B - 50bp DNA ladder;
C - 100bp DNA ladder;
MS - Mucking;
OS - Odcombe;
BSD - Brogborough 18m sample;
BSS - Brogborough 3m sample;
PL - Poyle;
HL - Hermitage leachate sample;
HS - Hermitage excavated refuse sample;
N - negative control (no template);
P - positive control (cloned *mcrA* PCR product)

For the landfill leachate samples, PCR products were generated directly from DNA prepared by the phenol/chloroform method (section 2.3.3). DNA extracted from the model landfill reactor leachate required further purification and concentration (section 2.4.3), in order to generate sufficient product for successful cloning. Clone libraries of greater than 100 clones were needed for the PCR-RFLP analyses. DNA extracted directly from the excavated refuse sample from the Hermitage landfill, required further purification (section 2.4.3), and two rounds of amplification in order to generate sufficient product for successful cloning. This may have been due to a low density of methanogens in the excavated refuse sample from the Hermitage landfill, or to inhibition of the PCR by impurities that were co-purified with the DNA.

Clone libraries were generated from the *mcrA* gene fragments amplified from each of the landfill samples. The clone libraries were screened by restriction analysis as described in the following sections, and representative clones were sequenced as described in chapter 4.

3.2.3 PCR-RFLP analysis

Clone libraries of *mcrA* PCR products were screened by restriction fragment length polymorphism (RFLP) analysis. The aim was to develop a method by which the diversity of the methanogen community in landfill could be determined. In addition, the RFLP screening was used to select clones for further analysis by nucleotide sequencing. The basic procedure for RFLP analysis involved amplifying the *mcrA* fragment from individual clones, digesting the PCR products with a restriction endonuclease, separating the digested fragments by gel electrophoresis, and comparing the banding patterns. RFLP patterns were used to define operational

taxonomic units (OTUs). OTU is a term that has been used in a number of studies to describe groups of clones that are related by a characteristic such as sequence similarity or RFLP patterns (Fernandez *et al*, 1999; Massana *et al*, 2000; Moeseneder *et al*, 1999; Moyer *et al*, 1994). Development of the RFLP screening method involved selection of a suitable restriction enzyme or enzymes, and determination of the optimum electrophoresis conditions to achieve sufficient resolution of the digested fragments.

3.2.3.1 Method development

Selection of restriction enzymes

The *mcrA* PCR product is relatively short (464 - 491bp). This means that endonucleases with recognition sites longer than four base pairs would not be expected to cut the PCR products frequently enough to provide a useful level of discrimination. Using the formula of Nei & Li (1979) it was calculated that a *mcrA* PCR product would contain between 0.90 and 1.92 cut sites for an endonuclease, such as *Taq*I (recognition site TCGA). This assumes a random nucleotide sequence and a G+C content in the range 23-62%. This is the G+C content determined for described methanogen species (Sowers, 1995). An endonuclease with a six base pair recognition sequence, such as *Eco*RI (recognition site GAATTC), would be expected to cut a *mcrA* PCR product between 0.06 and 0.13 times.

Tetrameric type II restriction endonucleases such as *Taq*I, cut DNA at specific sequences of four nucleotides. *Taq*I was used with cloned *mcrA* PCR products from the Odcombe sample. This analysis revealed 17 restriction fragment patterns in 102 clones. A second tetrameric restriction enzyme, *Rsa*I (GTAC) was used with the

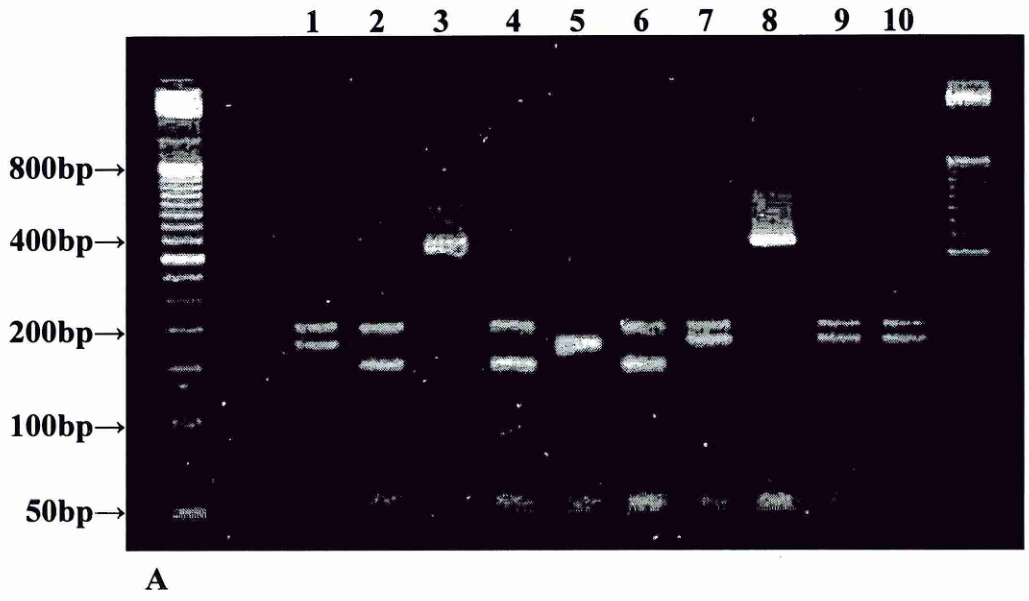
same clones to determine if the use of a second enzyme would significantly improve the discrimination of *mcrA* clones over that achieved with *Taq*I. Plate 3.3 shows the results of digesting ten *mcrA* PCR products separately with *Taq*I and *Rsa*I. The *Taq*I digest revealed four restriction fragment patterns (Plate 3.3a). The four patterns/groups consisted of lanes 1, 7, 9 and 10; lanes 2, 4 and 6; lanes 3 and 8; and lane 5. The *Rsa*I digest also revealed four patterns (Plate 3.3b). Lanes 1, 2, 6, 7 and 10 formed one group. Lane 3 formed a second group. Lanes 4, 5 and 9 formed a third group. Lane 8 formed the fourth group. Combining the results from the two enzymes gave seven groups from the ten cloned PCR products. Although using the two enzymes *Taq*I and *Rsa*I gave increased discrimination, it was decided that a sufficient level of discrimination was achieved with just *Taq*I. Using a single enzyme also made the method and analysis of results quicker and simpler.

Optimisation of gel electrophoresis

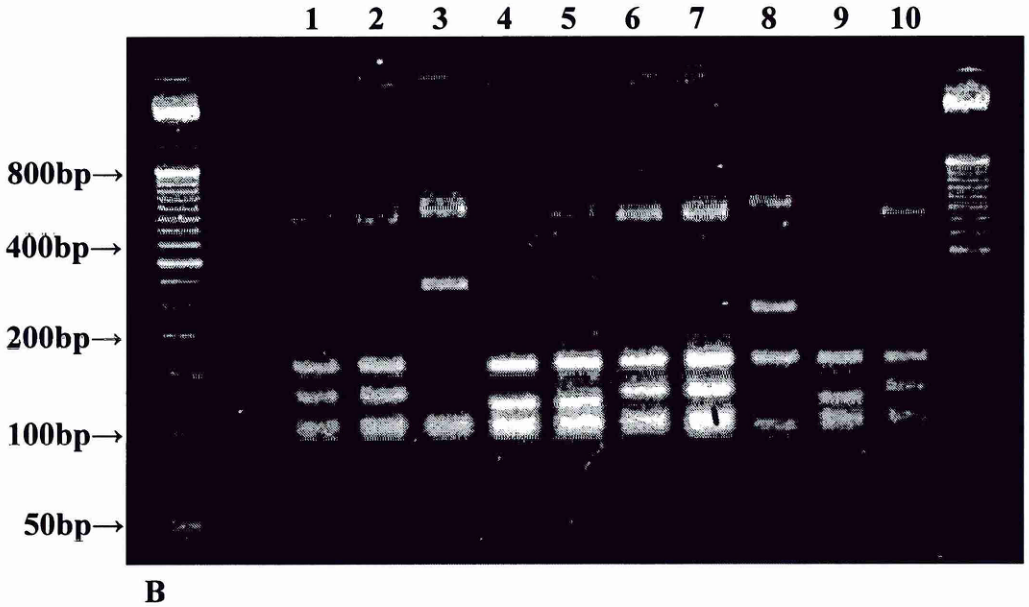
The fragments generated by *Taq*I digestion of *mcrA* PCR products range in size from 6 to 491 base pairs and some of the fragments differ in size by as little as two base pairs. Gel electrophoresis conditions were optimised for: 1) resolution of fragments in the range 6 to 491bp; 2) detection of the smaller fragments; and 3) accurate sizing of the fragments.

Electrophoresis of the *Taq*I digested PCR products was first performed on 2% agarose mini-gels (10×8×0.5cm), but these did not give sufficient resolution (Plate 3.4a). 2% agarose maxi-gels (25×20×0.5cm) were tried next and gave better resolution (Plate 3.4b). Separide (Gibco BRL) (2%) mini-gels were also tried and gave a similar level of resolution to 2% agarose maxi-gels (Plate 3.4c). Separide is a blend of

Plate 3.3 RFLP analysis of *mcrA* PCR products using *Taq*I and *Rsa*I.



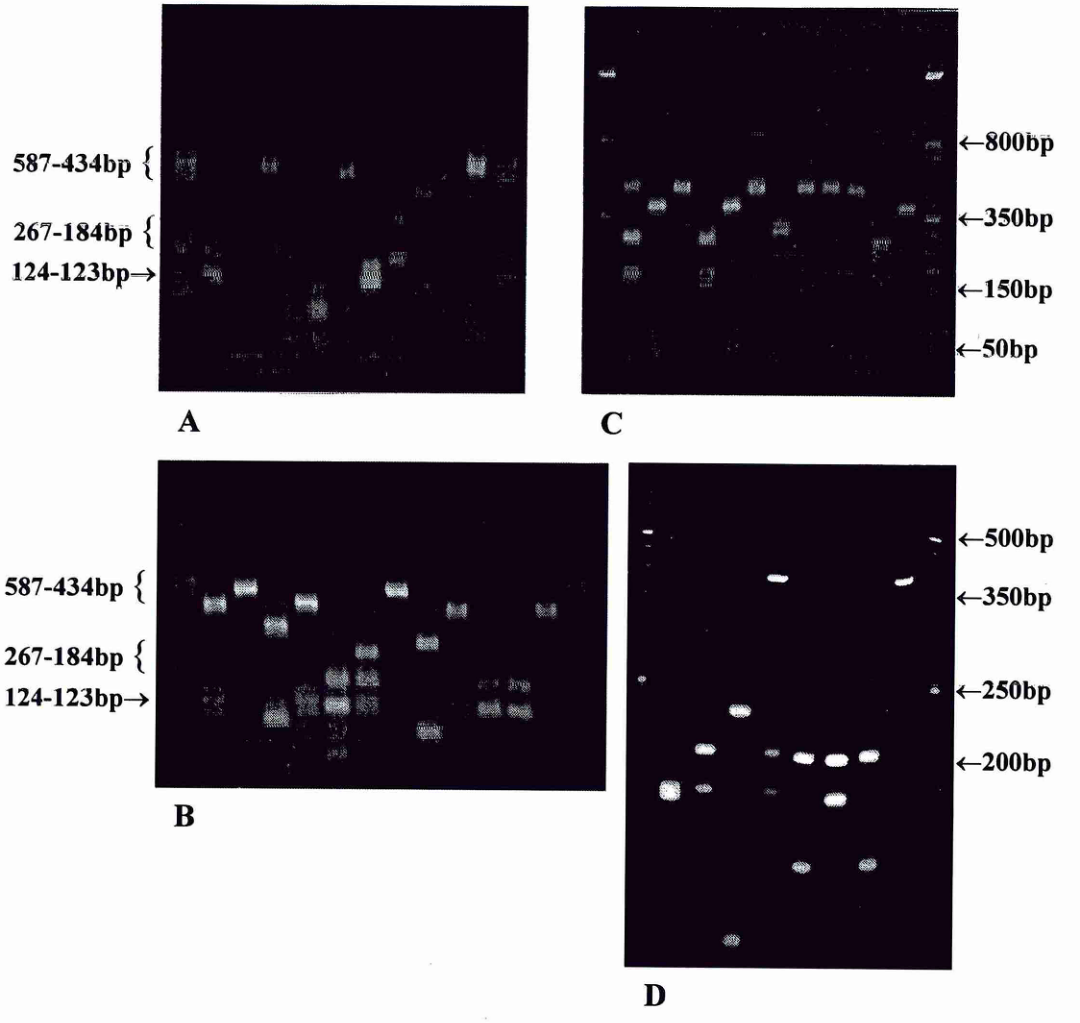
A



B

Legend: Ten cloned *mcrA* PCR products from the Brogborough 18m sample were digested separately with the tetrameric restriction endonucleases *Taq*I (A) and *Rsa*I (B). The resulting fragments were separated on a 4% (w/v) agarose gel, stained with ethidium bromide and visualised at 312nm. A 50bp DNA ladder was run in the outside lanes.

Plate 3.4 Optimisation of gel electrophoresis.



Legend: Several types of gel electrophoresis were tried to achieve optimum resolution of restriction fragments generated from *Taq*I digestion of *mcrA* PCR products. 4% agarose maxi-gels, as shown in Plate 3.5, gave the best results and were used routinely for RFLP analysis. The outside lanes of each gel contain molecular weight markers.

A - 2% agarose mini-gel (10×8×0.5cm);
B - 2% agarose maxi-gel (25×20×0.5cm);
C - 2% Separide mini-gel;
D - 6% acrylamide/bis-acrylamide (37:1) denaturing polyacrylamide gel (16×20×0.1cm).

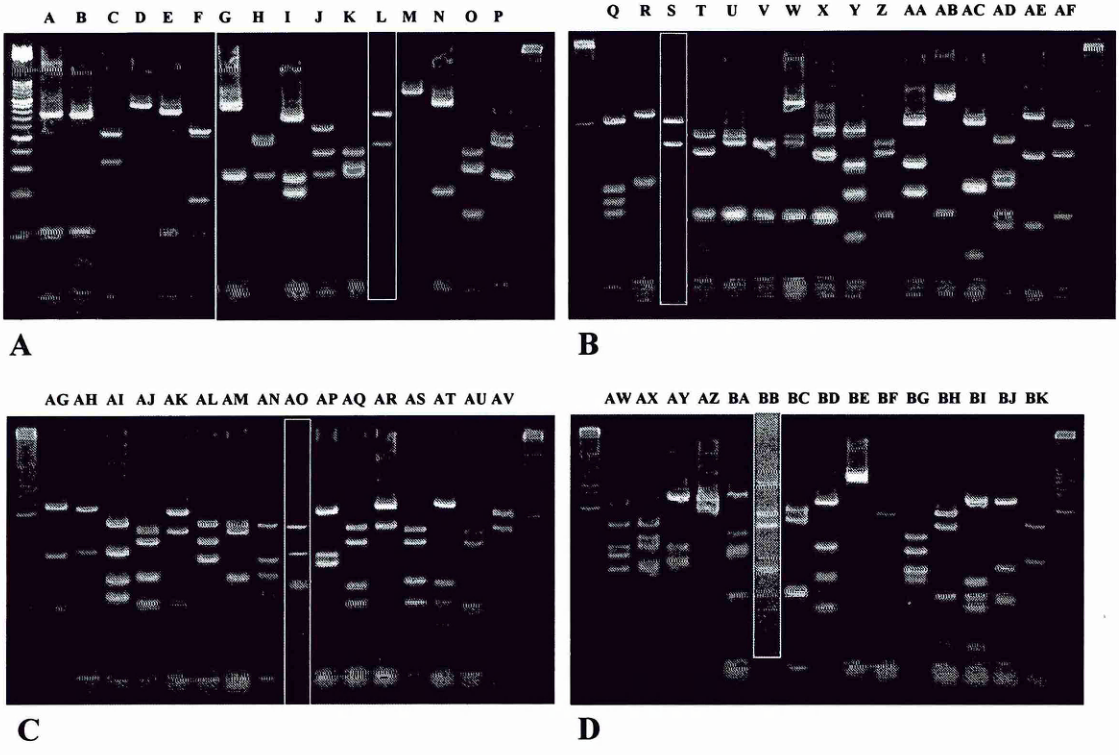
polysaccharides, which may be used to prepare gels in the same way as agarose. It is claimed to give resolution of fragments less than 500bp, equivalent to polyacrylamide gels. Denaturing polyacrylamide gels (16×20×0.1cm) were tried, and gave better separation of the fragments, but blurring of the bands and difficulty in detecting the smaller fragments was a problem (Plate 3.4d). In addition, polyacrylamide gels are more difficult and time-consuming to prepare than agarose gels; also, more complex and expensive electrophoresis apparatus is required. This is an important consideration if the PCR-RFLP method was to be applied to the routine monitoring of methanogen populations. Agarose maxi-gels (4%) proved to give the best results and these were used routinely for the RFLP analyses. Thirty clones could be analysed per gel.

The sizes of the fragments were initially estimated by comparison to a 50bp DNA ladder. Clones with different fragment patterns were then sequenced. The fragment sizes could then be determined precisely from the DNA sequence. Once a database of fragment sizes and patterns had been built up, it was possible to determine the majority of fragment sizes from the gels by comparison to the 50bp DNA ladder and fragments of known size on previous gels.

3.2.3.2 RFLP analysis of cloned *mcrA* PCR products from landfill

A total of 632 cloned *mcrA* PCR products from seven landfill samples were screened by RFLP analysis with *Taq*I. 63 different restriction fragment patterns (RFPs) were observed (Plates 3.5a-d). Each *mcrA* PCR product contained between zero and five *Taq*I cut sites. The majority contained either two or three cut sites. Clones sharing the same RFP were defined as an OTU. Table 3.2 shows the number of clones

Plate 3.5 Restriction Fragment Patterns (RFPs) detected in landfill by *Taq*I digestion of cloned *mcrA* PCR products.



Legend: Samples of excavated refuse material and leachate were taken from five landfill sites around England. DNA was extracted from the samples and a ~500bp fragment of the methanogen specific *mcrA* gene amplified by PCR. Clone libraries of the *mcrA* PCR products were generated. *McrA* PCR products amplified from the clones were digested with the tetrameric restriction endonuclease, *Taq*I. The resulting fragments were separated by agarose gel electrophoresis to reveal the RFPs. The molecular size marker is a 50bp DNA ladder with the bright band corresponding to 250bp. The lane labels correspond to the OTU numbers shown in Table 3.3.

Table 3.2 RFLP analysis of *mcrA* clone libraries from seven landfill samples.

Landfill samples	**MS[a]**	**OS[b]**	**BSD[c]**	**BSS[d]**	**PL[e]**	**HL[f]**	**HS[g]**	**Total**
No. of clones analysed	63	102	89	80	135	110	53	632
No. of OTUs detected [h]	6	17	10	15	31	15	14	63
Diversity index [i]	0.10	0.17	0.11	0.19	0.23	0.14	0.26	

Key: [a]Mucking, [b]Odcombe, [c]Brogborough - depth 18m, [d]Brogborough - depth 3m, [e]Poyle leachate, [f]Hermitage leachate, [g]Hermitage excavated refuse.
[h] Number of *Taq*I restriction fragment patterns detected.
[i] Number of OTUs detected divided by number of clones analysed.

analysed from each clone library and the number of OTUs identified. A diversity index was calculated for each landfill sample by dividing the OTUs by the number of clones analysed (Table 3.2). This appeared to indicate that the clone library generated from the Hermitage excavated refuse sample had the greatest diversity of OTUs. The clone library generated from the enrichment culture inoculated with leachate from the model landfill reactor containing material from the Mucking sample appeared to have the lowest diversity. Sequencing of several clones from each OTU was used to determine precisely the size of the restriction fragments generated with *Taq*I (Table 3.3). The total number of clones in each OTU is also shown in Table 3.3. The distribution of the *mcrA* clones among the OTUs for each landfill sample is shown in Figures 3.1a-g. Each population was dominated by two to four OTUs that accounted for 40-90% of the clones in each sample. The remainder of the population in each sample was made up of OTUs that were detected at a much lower frequency. Twenty-four OTUs were common to more than one sample, but only one OTU was detected in all seven samples. The remaining 39 OTUs were unique to different samples. Furthermore, 24 OTUs were represented by a single clone.

The *Taq*I RFPs were also determined for 44 described methanogen strains, plus 11 unidentified or incompletely described strains, from DNA sequences (Table 4.1, p148). Nine RFPs detected in the landfill samples matched RFPs from known methanogens (Table 4.1, p148). In addition, OTU T had the same *Taq*I RFP as a *mcrA* sequence detected in an anaerobic digester (Hougaard & Westermann, 2000). This indicated that the landfill samples might contain methanogen species related to *Methanobacterium formicicum*, *Methanobrevibacter ruminantium*, *Methanococcus jannaschii*, *Methanococcus igneus*, *Methanocorpusculum parvum*,

Table 3.3 OTUs and corresponding RFLPs of *Taq*I digested *mcrA* PCR products from seven landfill samples.

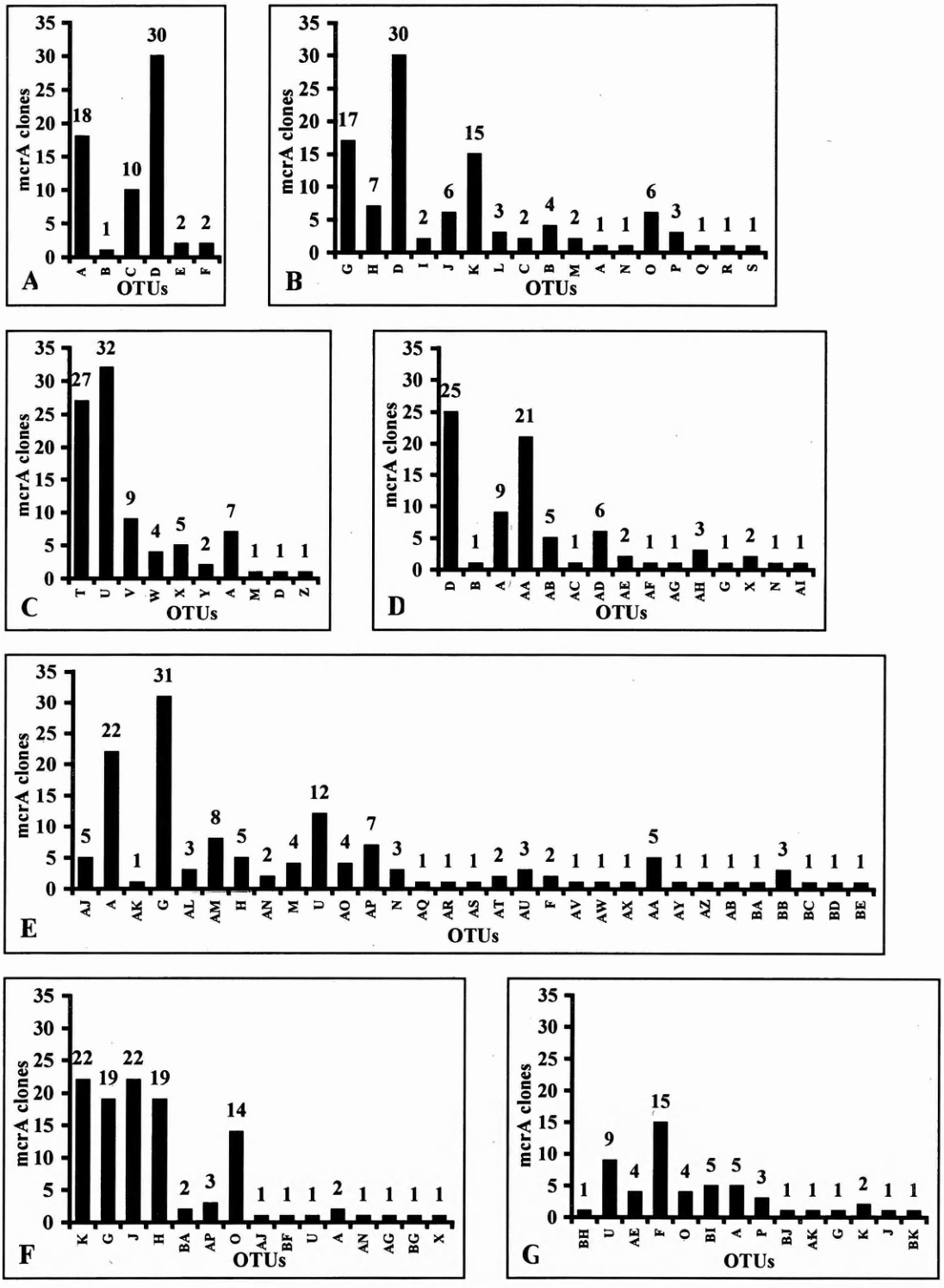
OTU	Sizes of DNA fragments (bp)		Total clones
	Cut with *Taq*I	Total	
A	51, 53, 387	491	64
B	33, 53, 384	470	6
C	9, 21, 170, 267	467	12
D	464	464	86
E	53, 417	470	2
F	45, 53, 93, 297	488	19
G	104, 387	491	69
H	104, 185, 202	491	31
I	77, 99, 297	473	2
J	104, 154, 233	491	29
K	104, 113, 120, 154	491	39
L	170, 294	464	3
M	491	491	7
N	77, 396	473	5
O	51, 53, 113, 120, 154	491	24
P	104, 184, 203	491	6
Q	6, 53, 67, 81, 263	470	1
R	86, 93, 288	467	1
S	21, 179, 267	467	1
T	30, 51, 53, 154, 203	491	27
U	51, 53, 184, 203	491	54
V	24, 51, 53, 179, 184	491	9
W	51, 53, 179, 208	491	4
X	51, 53, 154, 233	491	8
Y	39, 77, 126, 228	470	2
Z	24, 30, 51, 53, 154, 179	491	1
AA	77, 126, 267	470	26
AB	53, 438	491	6
AC	27, 84, 86, 267	464	1
AD	45, 53, 93, 105, 192	488	6
AE	45, 146, 297	488	6
AF	30, 53, 154, 254	491	1

OTU	Sizes of DNA fragments (bp)		Total clones
	Cut with *Taq*I	Total	
AG	51, 53, 120, 267	491	2
AH	21, 53, 126, 267	467	3
AI	59, 77, 126, 208	470	1
AJ	53, 83, 150, 184	470	6
AK	53, 184, 254	491	2
AL	113, 154, 203	470	3
AM	83, 184, 203	470	8
AN	67, 87, 113, 203	470	3
AO[a]	73, 79, 124, 194	470	4
AP	104, 120, 267	491	10
AQ	53, 73, 150, 194	470	1
AR	202, 289	491	1
AS	53, 79, 150, 188	470	1
AT	39, 53, 75, 303	470	2
AU	48, 51, 53, 154, 185	491	3
AV	203, 267	470	1
AW	83, 104, 120, 184	491	1
AX[a]	67, 104, 135, 185	491	1
AY[a]	77, 105, 306	488	1
AZ[a]	224, 267	491	1
BA	67, 104, 320	491	3
BB	83, 184, 224	491	3
BC	53, 202, 236	491	1
BD[a]	42, 53, 102, 267	464	1
BE	470	470	1
BF	77, 176, 220	473	1
BG	53, 67, 83, 117, 150	470	1
BH[a]	53, 184, 233	470	1
BI[a]	45, 53, 75, 297	470	5
BJ[a]	51, 53, 90, 297	491	1
BK[a]	45, 53, 75, 105, 192	470	1
Total			**632**

Key: Cloned *mcrA* gene PCR products were cut with the restriction endonuclease *Taq*I and the resulting fragments analysed by agarose gel electrophoresis. The fragment sizes were estimated against a 50bp DNA ladder and confirmed by analysis of the DNA sequences of representative clones from each OTU.

[a] Fragment sizes not confirmed by sequence analysis.

Figure 3.1 Distribution in OTUs of *mcrA* PCR products cloned from seven landfill samples.

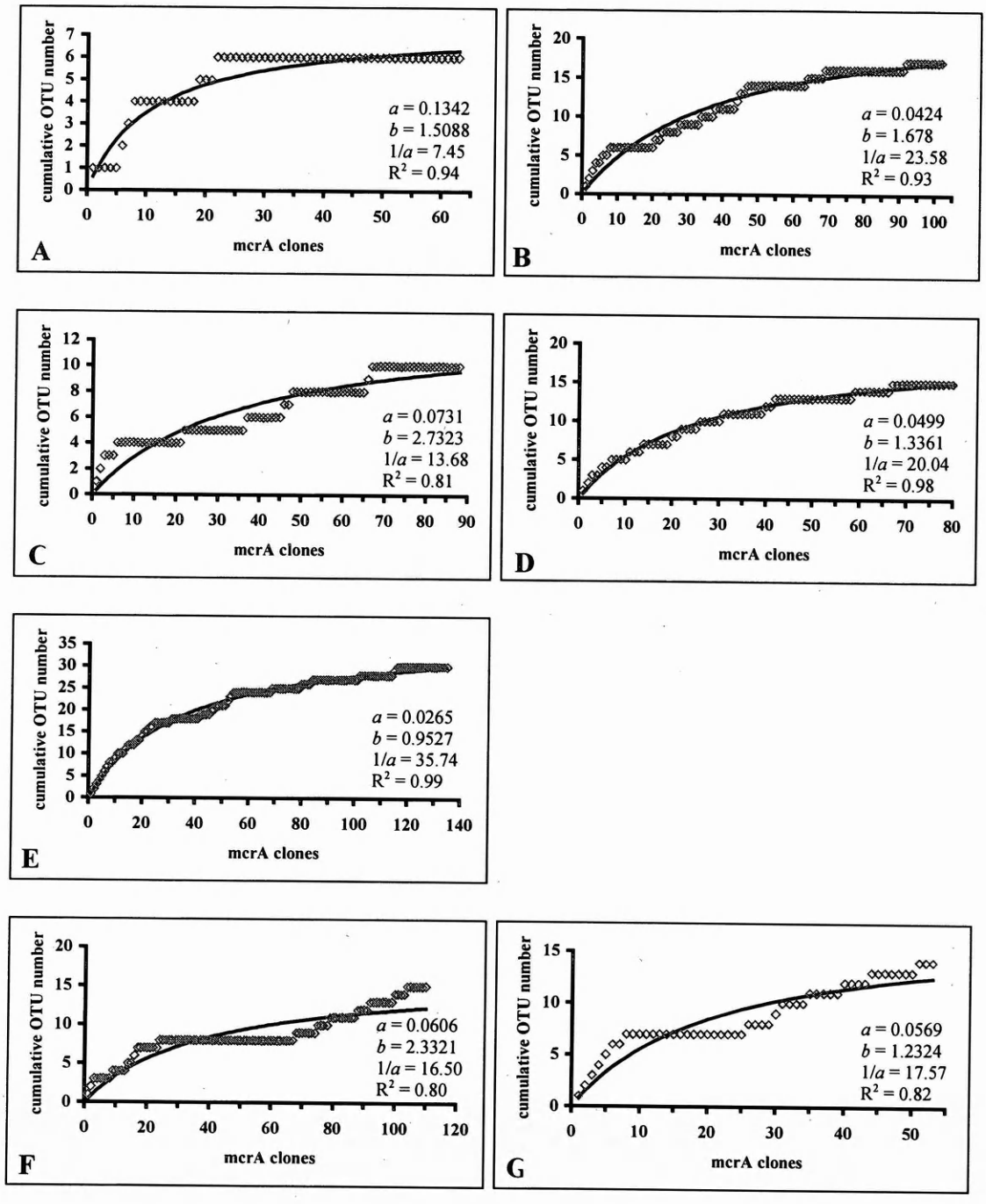


Legend: The distribution of *mcrA* clones in each landfill sample is displayed in a separate graph: Mucking (A), Odcombe (B), Brogborough 18m (C), Brogborough 3m (D), Poyle (E), Hermitage leachate (F), Hermitage excavated refuse sample (G). Abundance, as determined by the number of *mcrA* clones found in each OTU, was used to define the community structure. The OTUs are shown in the order of initial detection in each sample.

Methanocorpusculum aggregans, *Methanocorpusculum bavaricum*, *Methanoculleus bourgensis*, *Methanoculleus thermophilus*, *Methanofollis liminatans*, *Methanospirillum hungatei*, *Methanosarcina acetivorans*, *Methanosarcina barkeri*, *Methanosarcina mazei*, *Methanosarcina siciliae*, *Methanosarcina thermophila* and *Methanosaeta concilii* strain VeAc9. The list includes more than ten species because five of the ten RFPs were common to more than one species of methanogen. *M. formicicum* and *M. jannaschii* shared the same RFP, as did *M. ruminantium*, *M. igneus* and *M. concilii* VeAc9. In both these cases, the *mcrA* gene fragments did not contain any *Taq*I restriction sites and consequently the RFPs consisted of a single fragment of 464bp or 470bp. The other three RFPs common to more than one methanogen species were shared by closely related species. *M. parvum* and *M. aggregans* shared the same RFP, as did *M. liminatans* and *M. hungatei*, and four species of *Methanosarcina*. To identify the methanogen species giving rise to the other OTUs, clones from each OTU were sequenced and the phylogenetic affiliations of the sequences determined. The results of the sequencing and phylogenetic analysis are described in chapter 4. The phylogenetic analysis revealed that the *M. formicicum* sequence having the same RFP as OTU D was the *mrtA* gene, which encodes subunit A of the isoenzyme, MCRII, of methyl coenzyme M reductase.

To determine whether the methanogen diversity present in each sample was well described by the *mcrA* clones examined, the cumulative number of OTUs was plotted as a function of clone number (Figure 3.2). The *mcrA* clones were arranged in the order of detection. This method has been used by Chin *et al* (1999), Godon *et al* (1997a), Moyer *et al* (1994) and Sekiguchi *et al* (1998). Figure 3.2a shows that

Figure 3.2 Plot of cumulative number of OTUs as a function of clone number for seven landfill samples.



Legend: To determine if the diversity of methanogenic *Archaea* in the landfill samples was fully represented by the *mcrA* clones analysed, the cumulative number of OTUs was plotted against the *mcrA* clones in the order of detection. The plots were fitted to a parabolic function $\{y = x/(ax + b)\}$, where y = cumulative number of different OTUs, x = number of clones analysed and a, b = coefficients. $1/a$ = estimated total number of different OTUs. Landfill samples: Mucking (A), Odcombe (B), Brogborough 18m (C), Brogborough 3m (D), Poyle (E), Hermitage leachate (F), Hermitage solid sample (G).

with the Mucking sample after the first 22 clones were examined all six OTUs had been detected and no additional OTUs were detected among the remaining 41 clones. This appeared to indicate that the diversity in the Mucking enrichment culture was well described. However, in the Hermitage leachate sample a similar plateau was seen (Figure 3.2f) after eight OTUs were detected, but a further seven OTUs were detected in the last 43 clones. One-hundred-thirty-five clones were analysed from the Poyle sample and a definite levelling off in the rate of detection of new OTUs was observed (Figure 3.2e). The curves were fitted to a parabolic function, $y = x/(ax + b)$, where x = number of clones, y = cumulative number of different OTUs and a, b = coefficients. This formula was used by Sekiguchi *et al* (1998) to estimate 16S rRNA sequence diversity in anaerobic digesters. R^2 values were calculated for each curve, to show the goodness of fit of the curves to the data points. All the R^2 values were ≥ 0.8 indicating a good fit in all cases. The possible total number of OTUs in each landfill sample was estimated as $y = 1/a$, when x = infinity. According to the calculations, the possible total number of OTUs was estimated to be approximately 7, 24, 14, 20, 36, 17 and 18 for the Mucking, Odcombe, Brogborough 18m, Brogborough 3m, Poyle, Hermitage leachate and Hermitage excavated refuse samples respectively. This method indicated different relative levels of diversity between the samples compared to the diversity indices shown in Table 3.2. Based on the number of OTUs detected divided by the predicted number of OTUs, it was estimated that between 71% and 88% of the diversity present in the clone libraries was detected, as measured by the PCR-RFLP method.

3.2.4 Denaturing gradient gel electrophoresis

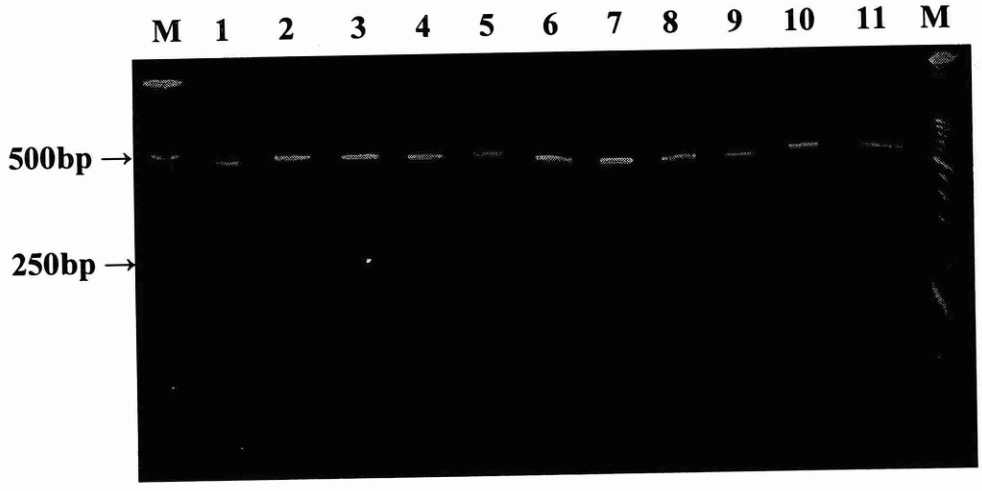
The objective was to develop DGGE of the *mcrA* PCR product as a rapid measure of methanogen diversity. DGGE would allow differences in the structure of the methanogen population between samples to be visualised rapidly without the need for cloning.

In DGGE, DNA fragments of the same length, but with different nucleotide sequences can be separated. Separation is based on the electrophoretic mobility of a partially melted double stranded DNA molecule in a polyacrylamide gel containing a linearly increasing gradient of DNA denaturants (urea and formamide). Muyzer *et al* (1996) and Silvey and Blackall (1995) have described the theory behind DGGE and its application to the investigation of microbial diversity.

The *mcrA* PCR products vary in size from 464 base pairs to 491 bp. Experiments were carried out to determine the effect this variation might have on the migration of the PCR products under DGGE conditions. PCR products of known size were electrophoresed on a polyacrylamide gel with denaturing conditions that completely melted the double stranded DNA molecules. A small difference was observed in the distance migrated by the *mcrA* PCR products, which appeared to correlated with the sizes of PCR products (Plate 3.6).

Perpendicular denaturing gradient gel electrophoresis (DGGE) (where the denaturing gradient is perpendicular to the direction of electrophoresis) was performed to determine the optimum gradient of denaturants for maximum resolution of the PCR products. The electrophoretic pattern on a perpendicular denaturing gradient gel will

Plate 3.6 Denaturing PAGE of *mcrA* PCR products from described methanogen species.



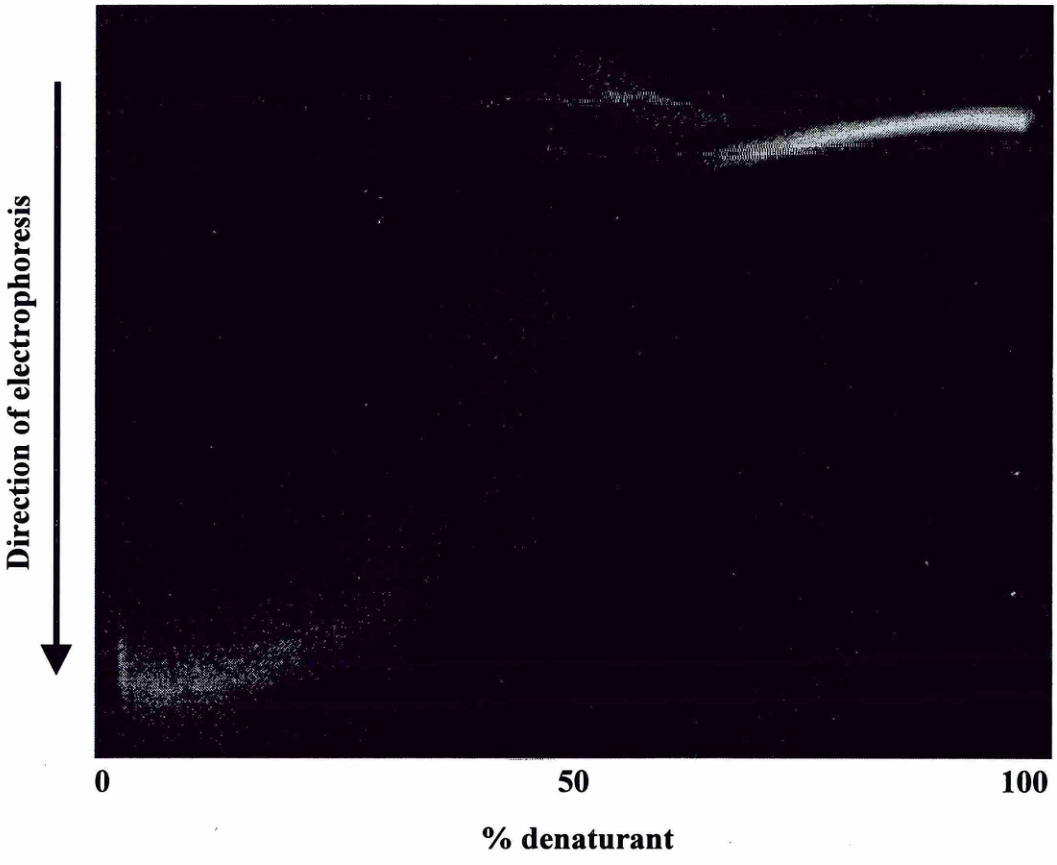
Legend: *mcrA* PCR products, amplified from the methanogen species listed, were loaded on a denaturing polyacrylamide gel (6% (w/v) acrylamide/bis-acrylamide 37:1; 42% (w/v) urea; 40% (v/v) deionised formamide). The gel was electrophoresed at 100 volts, 60°C for 4 hours, then stained with SYBR Green I, and the DNA visualised at 312nm.

Lanes: M) 50bp DNA ladder,
1) *Methanopyrus kandleri* (467bp),
2) *Methanohalophilus halophilus* (488bp),
3) *Methanosarcina mazei* (488bp),
4) *Methanospirillum hungatei* (491bp),
5) *Methanoculleus bourgensis* (491bp),
6) *Methanobacterium espanolae* (467bp),
7) *Methanobacterium bryantii* (467bp),
8) *Methanobrevibacter arboriphilicus* (467bp),
9) *Methanobrevibacter ruminantium* (470bp),
10) *Methanocorpusculum parvum* (491bp),
11) *Methanocorpusculum bavaricum* (491bp).

look like a sigmoid-shaped curve. DNA molecules at the left side of the gel, where the denaturant concentration is low, will migrate as double stranded DNA. On the other side of the gel, where the denaturant concentration is high, the molecules will melt into branched molecules as soon as they enter the gel, and will migrate extremely slowly. A steep transition in mobility occurs at the denaturant concentration that corresponds to the melting temperature of the lowest melting domain of the fragment (Muyzer *et al*, 1996). A mixture of 12 *mcrA* PCR products cloned from the Mucking and Odcombe landfills, and representing eight OTUs, were subject to perpendicular DGGE (Plate 3.7). The pattern of the PCR products in Plate 3.7 show a sharp inflexion at about 50% denaturant, which corresponds to the melting temperature of the lowest melting domain. The optimum denaturant gradient for resolution of the DNA fragments corresponds to the steepest part of the curve on the perpendicular denaturing gradient gel. For the *mcrA* PCR products in Plate 3.7, this is between 45% and 55% denaturant.

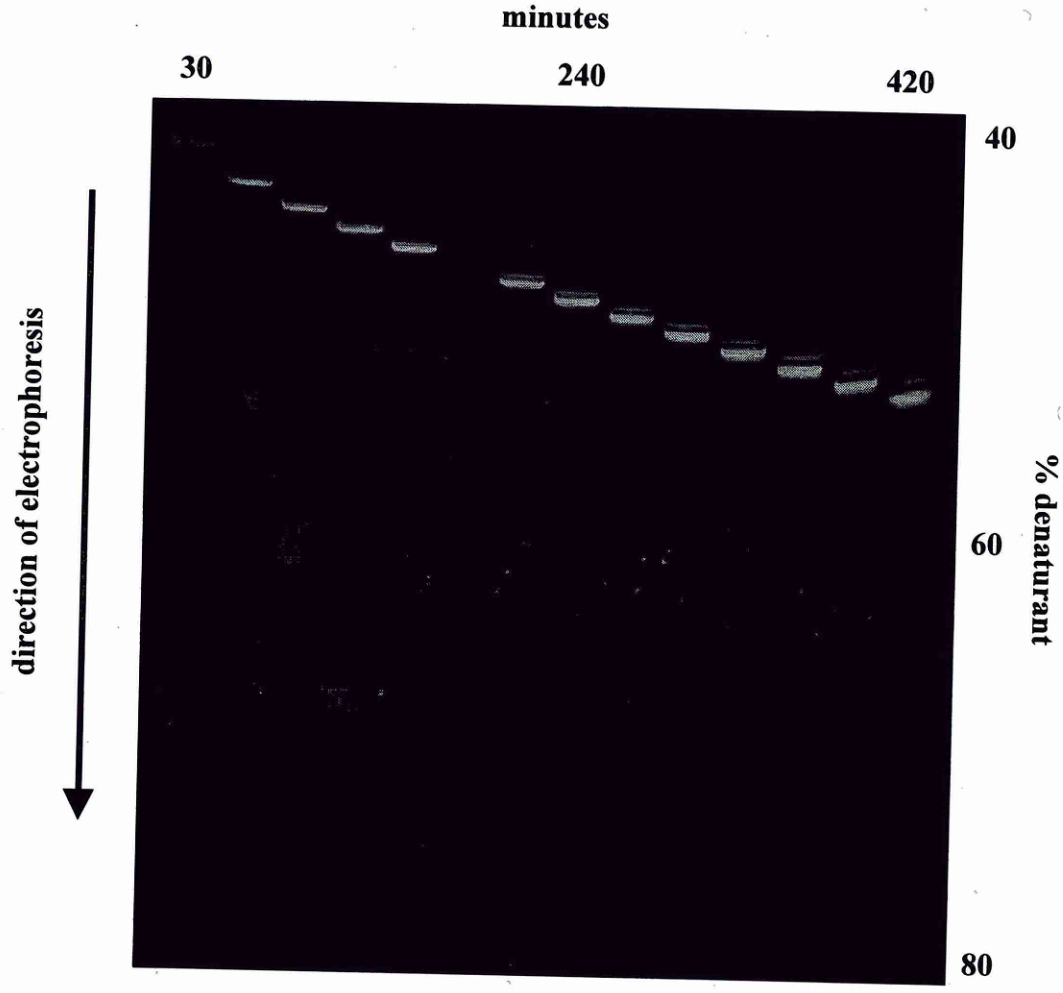
A series of parallel DGGE time-travel gels were run to establish the optimum denaturant concentration. The concentration range of denaturant on the gels was gradually narrowed down in an attempt to find the denaturant concentration and electrophoresis time that would give maximum resolution of the PCR products. Initial results looked promising with the PCR products starting to resolve (Plate 3.8). The PCR products were resolving at denaturant concentrations between 35% and 60%. However, resolution of the PCR products sufficient to allow complex mixtures of *mcrA* genes from many different species to be resolved was not achieved.

Plate 3.7 Perpendicular DGGE of *mcrA* PCR products.



Legend: A mixture of 12 *mcrA* PCR products cloned from the Mucking and Odcombe landfills were electrophoresed on a polyacrylamide gel (10% (w/v) acrylamide/*bis*-acrylamide (37:1)) with a denaturing gradient of 0-100% (21-29.4% (w/v) urea, 20-28% (v/v) formamide), perpendicular to the direction of electrophoresis. The gel was run at 56°C, 130 volts for 2 hours. The gel was stained with ethidium bromide and the DNA visualised at 312nm.

Plate 3.8 DGGE time-travel experiment to determine the optimum denaturant concentration for resolution of *mcrA* PCR products.

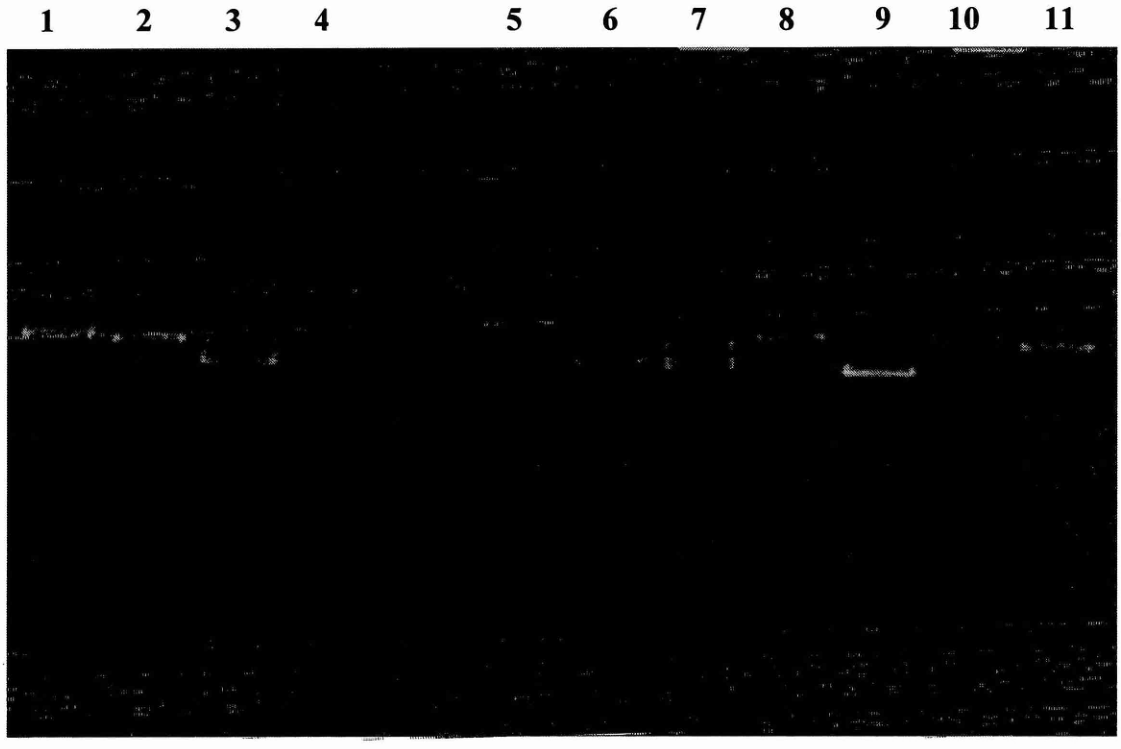


Legend: A mixture of *mcrA* PCR products was prepared by pooling amplification reactions of *mcrA* from eight enrichment cultures of landfill methanogens. The pooled PCR products were loaded on the gel at 30-minute intervals. The polyacrylamide gel with a parallel denaturant gradient of 40-80% was run at 130 volts, 60°C for 7 hours. The gel was stained with SYBR Green I and viewed on an UV transilluminator (312nm).

The related technique of temporal temperature gradient electrophoresis (TTGE) was also evaluated. In TTGE, the denaturing gradient is provided by increasing the buffer temperature gradually over the course of the electrophoresis. The concentration of urea and/or formamide is constant throughout the gel to lower the melting temperature of the DNA molecules. TTGE of *mcrA* PCR products from known methanogen species resulted in some resolution of the gene fragments (Plate 3.9). Again, the level of separation achieved was not enough for a mixture of PCR products from different species to be resolved in a single sample.

An isoenzyme of methyl CoM reductase is present in some methanogens (Bonacker *et al*, 1993; Bult *et al*, 1996; Lehmacher & Klenk, 1994). The gene encoding the isoenzyme MCRII, referred to as *mcr*II or *mrt*, is phylogenetically distinct from *mcr*I, and has only been detected in members of the *Methanobacteriales* and *Methanococcales* (Reeve *et al*, 1997b; Springer *et al*, 1995). A PCR product was generated from a pure culture of *Methanobacterium bryantii* using the *mcrA* primers. When the PCR product was subject to TTGE, two bands were resolved (Plate 3.9). Two bands were also resolved from a PCR product generated from a pure culture of *Methanobacterium espanolae*. It was concluded that these two bands must be *mcrA* and *mrtA*, both of which had been amplified. PCR products generated from pure cultures of *Methanobrevibacter arboriphilicus, Methanobrevibacter ruminantium, Methanospirillum hungatei, Methanoculleus bourgensis, Methanocorpusculum bavaricum, Methanocorpusculum parvum, Methanohalophilus halophilus, Methanosarcina mazei* and *Methanosaeta concilii* were also analysed by TTGE. A single band was visible on the gel for each of these species. This may be because

Plate 3.9 TTGE of *mcrA* PCR products from described species of methanogens.



Legend: *McrA* PCR products from the species listed were electrophoresed on a polyacrylamide gel (6% (w/v) acrylamide/*bis*-acrylamide (37:1), 42% (w/v) urea, 20% (v/v) formamide) at 100 volts for 17 hours with a temperature ramp rate of 0.3°C hr^{-1} and the temperature range from 45°C to 50°C.

Lanes: 1) *Methanohalophilus halophilus*,
2) *Methanospirillum hungatei*,
3) *Methanobacterium espanolae*,
4) *Methanosaeta concilii*,
5) *Methanoculleus bourgensis*,
6) *Methanobrevibacter arboriphilicus*,
7) *Methanobacterium bryantii*,
8) *Methanocorpusculum parvum*,
9) *Methanobrevibacter ruminantium*,
10) *Methanocorpusculum bavaricum*,
11) *Methanosarcina mazei*.

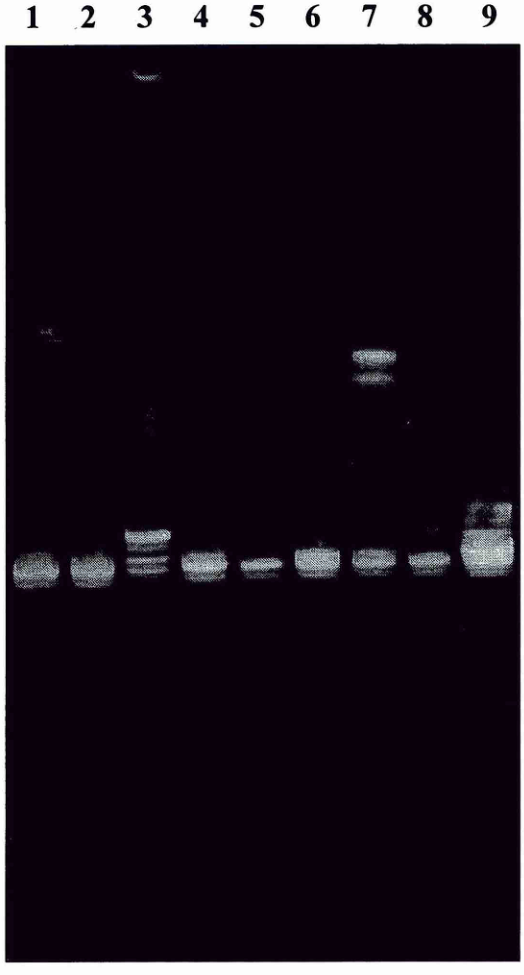
these species do not contain the *mrtA* gene, or the *mrtA* gene from these species is not amplified by the *mcrA* primers.

The implication of the amplification of *mrtA* for DGGE analysis is that the diversity of a methanogen community could be overestimated because two genes are amplified from some species. This is analogous to the problem of multiple heterogeneous ribosomal RNA operons in some *Bacteria* (Farrelly *et al*, 1995; Stackebrandt *et al*, 1998).

Plate 3.10 shows the results of parallel DGGE of *mcrA* PCR products generated from nine enrichment cultures inoculated with a 10^{-12} dilution of leachate from a model landfill reactor, containing material from the Odcombe landfill. Under the conditions employed, separate bands were resolved in all of the samples. This is particularly clear in lane 3, where five bands can be distinguished. However, the diversity of the methanogen population in the enrichment cultures would be expected to be low. The PCR-RFLP analysis indicated that the landfill samples contained diverse methanogen communities. In the case of the Odcombe sample, 17 OTUs were detected by PCR-RFLP analysis. It seems unlikely that 17 or more bands would be distinguishable with the resolution obtained for the DGGE.

The addition of a GC-clamp to PCR products has been shown to increase the number of sequence variants that can be resolved (Muyzer *et al*, 1993). A GC-clamp is a 30 to 40bp GC-rich sequence added to the 5' end of one of the PCR primers and incorporated into the products during PCR amplification. The GC-clamp becomes the highest temperature melting domain in the PCR products. This makes sequence

Plate 3.10 Parallel DGGE of *mcrA* PCR products amplified from model landfill reactor leachate dilution cultures.

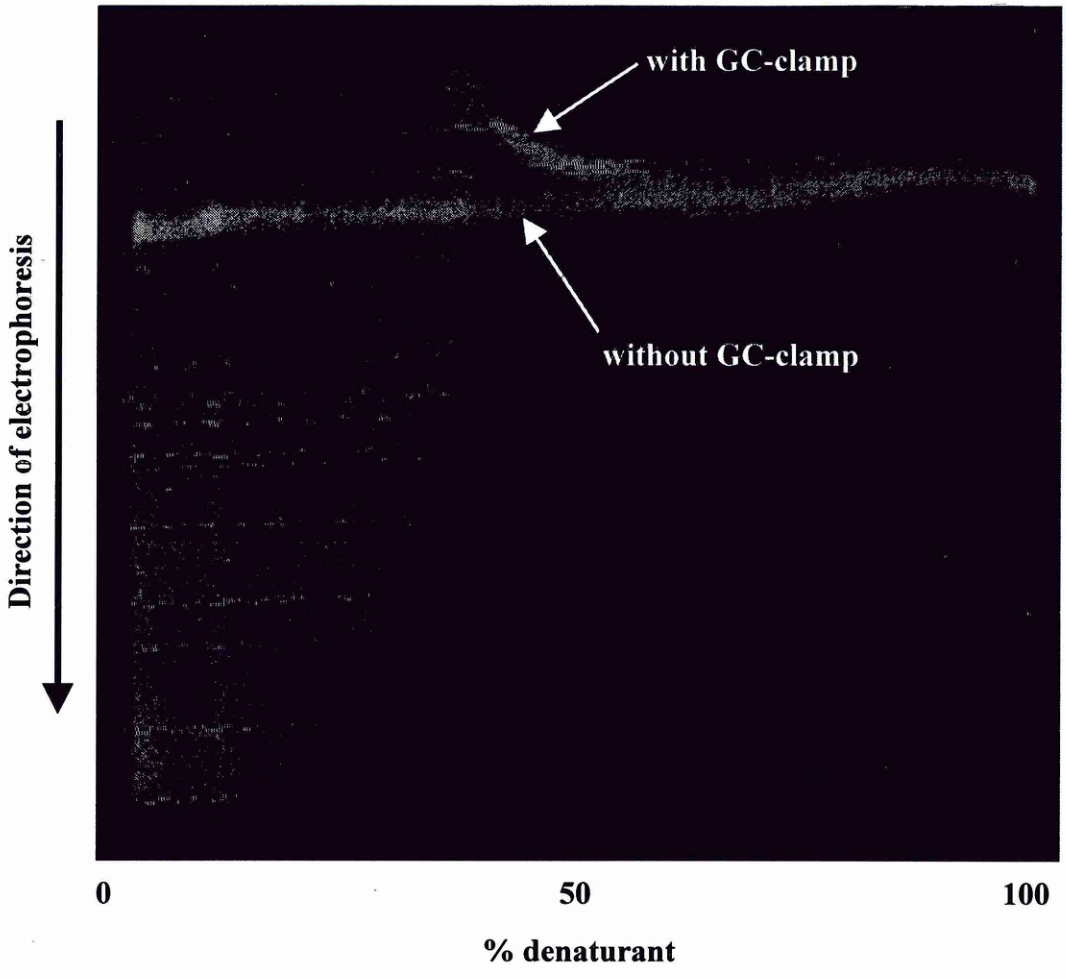


Legend: *mcrA* PCR products were amplified from enrichment cultures inoculated with a 10^{-12} dilution of leachate from a model landfill reactor, containing material from the Odcombe landfill. The PCR products were electrophoresed on a polyacrylamide gel (6% (w/v) acrylamide/*bis*-acrylamide (37:1)) with a denaturing gradient of 50-70% (21-29.4% (w/v) urea, 20-28% (v/v) formamide) at 60°C, 100 volts for 15 hours 45 minutes. The gel was stained with SYBR Green I and the DNA visualised at 312nm.

Lanes: 1) enrichment culture 24b,
2) enrichment culture 25b,
3) enrichment culture 27b,
4) enrichment culture 32b,
5) enrichment culture 34b,
6) enrichment culture 35b,
7) enrichment culture 37b,
8) enrichment culture 38b,
9) enrichment culture 39b.

variation in other high temperature melting domains accessible to DGGE analysis, by stabilising the molecules and preventing them separating into single stranded DNA. A 40bp GC-rich sequence was added to the *mcrA* reverse primer, as described in section 2.14. The reverse primer was chosen because it was the shorter of the two *mcrA* primers. A clear difference was observed in the melting characteristics of *mcrA* PCR products with and without the GC-clamp on perpendicular DGGE (Plate 3.11). However, the use of the GC-clamp in parallel DGGE did not improve the resolution of the PCR products.

Plate 3.11 Perpendicular DGGE of *mcrA* PCR products with and without a GC-clamp



Legend: A cloned *mcrA* PCR product from the Odcombe landfill was amplified with and without the GC-clamp, as described in section 2.14. The PCR products were mixed and electrophoresed on a polyacrylamide gel (10% (w/v) acrylamide/*bis*-acrylamide (37:1)) with a denaturing gradient of 0-100% (21-29.4% (w/v) urea, 20-28% (v/v) formamide), perpendicular to the direction of electrophoresis. The gel was run at 60°C, 200 volts for 2.5 hours. The gel was stained with ethidium bromide and the DNA visualised at 312nm.

3.3 DISCUSSION

3.3.1 Landfill samples

It is well established that landfills are extremely heterogeneous and consequently it is probably not possible to obtain a truly representative sample. Barlaz (1997) suggested that by collection of numerous samples from different parts of a landfill, one can obtain some indication of the status of a landfill. The excavated refuse samples used in this study, whilst not being representative of the microbial community in the whole landfill, were sufficient for the development of molecular techniques. A sample of leachate, which percolates through the landfill material, was believed likely to yield a more representative sample of the microbial community. Previous studies have used samples of excavated or fresh refuse (Barlaz *et al*, 1989; Fielding *et al*, 1988; Ladapo and Barlaz, 1997; Palmisano *et al*, 1993). Luton (1996) used oligonucleotide probes to study methanogens in both excavated refuse samples and leachate, but did not report any difference in the results from the two types of sample. To determine if a leachate sample was more representative than an excavated refuse sample, we obtained both types from the same landfill, for comparison of the methanogen populations. It was anticipated that the excavated refuse sample would contain the same or a sub-population of the methanogens detected in the leachate sample. However, comparison of the excavated refuse and leachate samples from the Hermitage landfill by restriction analysis, detected 15 restriction fragment patterns (RFPs) in the leachate and 14 in the solid sample, only 5 of which were common to both samples, Table 3.2. Comparison of the methanogen populations in each sample is discussed in more detail later.

3.3.2 DNA isolation, PCR amplification and cloning of *mcrA* gene fragments

The methodology employed in this study has enabled the isolation of DNA and the amplification of the desired gene fragments by PCR, from both leachate and excavated refuse samples directly, without the need for any enrichment. Isolation of DNA directly from excavated refuse samples avoids any selective enrichment that might occur in the accelerated model landfill reactors. DNA was isolated directly from the excavated refuse sample from the Hermitage landfill for this reason. The model landfill reactors were used to encourage the growth of an active methanogen population, without the selection imposed by growth in a defined medium. The model landfill reactors also allowed a liquid sample to be obtained from the excavated refuse material, from which it would be easier to isolate DNA. Improvements in the DNA extraction methodology meant this step could be omitted. However, it did prove more difficult to amplify the *mcrA* genes after direct isolation of DNA compared with isolation of DNA from model landfill reactor leachate. This difficulty may have been due to the nature of the Hermitage sample, which was drier and less decomposed than the other samples and therefore likely to contain fewer methanogens. Alternatively, the PCR may have been inhibited by impurities that were co-purified with the DNA. Inhibition of the PCR by contaminating compounds, such as humic acids, co-purified with DNA from soils and sediments has been reported (Smalla *et al*, 1993; Steffan *et al*, 1988). These inhibitory compounds may not have been leached from the refuse by the liquid percolating through the reactors. Nevertheless, direct isolation of DNA from solid landfill samples and amplification of the target gene is possible and would be the method of choice to avoid any enrichment bias.

A second round of amplification with nested-PCR primers could be used to increase

the sensitivity of the amplification step. Daly *et al* (2000) used both direct and nested-PCR to amplify the 16S rDNA of sulfate-reducing bacteria from landfill leachate. They found that with certain samples and primers, PCR products were generated by nested-PCR, but not by direct PCR. Daly (2000) observed extra bands in TTGE profiles generated from nested-PCR products compared to direct PCR products amplified with the same primers and templates. The wealth of sequence data for the *mcrA* gene obtained in this study (chapter 4) could be used to design a second pair of primers internal to the existing *mcrA* primers. Nested-PCR is preferable to a second round of amplification with the original primers, which can lead to the generation of non-specific products.

Bias in multi-template PCR amplifications and chimera formation has been observed by a number of workers, as described in section 1.4.4.2. The first five cycles of the PCR included a temperature ramp of 0.1°C sec^{-1} between the annealing and extension steps. The temperature ramp allowed time for the degenerate primers to be extended and to form a more stable hybrid before the temperature reached 72°C. This step may have helped to reduce the bias leading to preferential amplification of templates that formed more stable hybrids with the primers. In addition, a long extension time (2 minutes) was used to ensure complete primer extension. Premature termination of primer extension has been implicated as a cause of chimera formation (Paabo *et al*, 1990).

3.3.3 PCR-RFLP analysis

The investigation of microbial diversity in nature by PCR-RFLP analysis is a popular strategy because of its simplicity and economy. The majority of studies that have

employed the PCR-RFLP method, have used ribosomal DNA as the target. The major advantages of ribosomal DNA are its ubiquity and the vast database of sequence information that is available. This store of sequence information facilitates the design of PCR primers specific for the group of organisms being studied, and it allows the selection of appropriate restriction enzymes for the RFLP analysis. Whitby *et al* (1999) used published sequences of 16S rDNA from ammonia-oxidising bacteria to select restriction enzymes for PCR-RFLP analysis. Moyer *et al* (1996) used a computer-simulated RFLP analysis of published small sub-unit (SSU) rRNA gene sequences, to identify the optimal combination of tetrameric restriction enzymes, for RFLP screening of cloned SSU rRNA genes from undefined bacterial clone libraries. The disadvantage of using 16S rDNA is that because it is highly conserved, three restriction enzymes in combination are frequently required to distinguish between closely related taxa (Moyer *et al*, 1996; Whitby *et al*, 1999). Furthermore, it can be difficult to target specific groups of organisms based on 16S rDNA.

The genes encoding enzymes can be specific markers for groups of organisms sharing a common phenotype, such as methane production. Functional genes have been the target of PCR-RFLP analyses (Braker *et al*, 2000; Darrasse *et al*, 1994). In many cases, functional genes provide a resolution below species level because of the higher evolutionary rates of the less conserved functional molecules. Additionally, the analysis of functional genes may indicate functional diversity in the environment (Braker *et al*, 2000). The *mcrA* gene has been used as a functional marker gene for the study of methanogens in rice field soil, the hindgut of termites, blanket bog peat, marine sediments and landfill (Edwards *et al*, 1998; Elberson & Sowers, 1997; Kudo *et al*, 1998; Lueders *et al*, 2001; Luton, 1996).

In this study, PCR-RFLP analysis of the *mcrA* gene provided a measure of the diversity of the methanogen community in landfill samples. The community diversity was indicated by the number of different OTUs in each sample. Sixty-three different OTUs were identified in the seven landfill samples analysed. The number of OTUs detected in each sample ranged from six to 31 (Table 3.2). The majority of OTUs were unique to a particular landfill sample. Only 24 OTUs were common to more than one sample, and just one was found in all seven samples. These results appeared to indicate that the diversity of methanogens in landfill was far greater than that demonstrated by previous studies. Previous studies have used either culture-based methods to isolate landfill methanogens (Finlay and Fenchel, 1991; Ladapo and Barlaz, 1997; Mori *et al*, 2000) or probes based on described methanogen species to identify methanogens in landfill (Fielding & Archer, 1986; Fielding *et al*, 1988; Luton, 1996). These studies have identified between six and ten species of methanogens in landfill. The PCR-RFLP method used in this study is not subject to the limitations of the methods used in the previous studies, and consequently the level of diversity detected was far greater.

Some caution must be used when comparing the diversity in each sample because the samples were not all treated in the same manner prior to PCR of the *mcrA* gene. Furthermore, some samples were of excavated refuse material while others were leachate. Nevertheless, it is interesting to note that the sample with the lowest diversity was the Mucking sample. Leachate from the model landfill reactor containing material from the Mucking landfill was used to inoculate an anaerobic culture from which DNA was later extracted. The Mucking sample was therefore subject to a selective bias in favour of those methanogen species able to grow best in

that culture medium. It is also of interest that the sample of excavated refuse material from the Hermitage landfill had a higher diversity index than the leachate sample from the same landfill. Landfill leachate results from liquid that percolates through the waste carrying microorganisms and other particles with it, until it accumulates at the bottom of the landfill. Leachate, because it has been in contact with a large amount of the waste, might be expected to contain a greater diversity of microorganisms, compared to a small sample of waste material. This does not appear to be the case for the samples from the Hermitage landfill. However, it is not possible to draw firm conclusions from the analysis of one leachate and one solid sample from a single landfill.

The RFLP analysis also provided information on the structure of the methanogen community in each landfill sample (Figure 3.1). The structure of the methanogen community can be viewed both, in terms of the OTUs present, and the relative frequency with which each OTU was detected. The structures of the methanogen communities in each landfill sample were clearly very different. Even samples obtained from different depths within the same borehole at the Brogborough landfill contained different methanogen communities. Twenty-two OTUs were detected in these two samples, but only three were common to both samples. In the case of the excavated refuse and leachate samples from the Hermitage landfill, six out of 23 OTUs were common to both samples. These results clearly demonstrate that landfills are extremely heterogeneous and single samples of either excavated refuse material or leachate are not representative of the microbial community in the whole landfill.

The identity of nine of the OTUs was established tentatively by determining the *Taq*I

RFPs of the *mcrA* genes from 44 described methanogen species. This indicated that the PCR-RFLP method had detected a number of methanogen species that had been detected previously in landfill. Namely, *Methanobacterium formicicum*, *Methanoculleus bourgensis*, *Methanospirillum hungatei*, *Methanofollis liminatans* and the genus *Methanosarcina*. Luton (1996) reported detecting *M. bourgensis* in landfill leachate using an oligonucleotide probe that hybridised to the *mcrA* genes from *M. bourgensis*, *M. hungatei* and *M. liminatans*. Therefore, it is not clear whether all three of these species have been detected in landfill. *M. hungatei* and *M. liminatans* shared the same RFP, while *M. bourgensis* had a different RFP. The RFP shared by *M. hungatei* and *M. liminatans* was detected in all seven landfill samples, while the *M. bourgensis* RFP was only detected in three samples.

In addition, species that had not been detected before in landfills were indicated by the PCR-RFLP results. In particular, the genus *Methanocorpusculum*, *Methanosarcina thermophila* and possibly, *Methanobrevibacter ruminantium*, *Methanococcus jannaschii* and *Methanococcus igneus*. However, *M. ruminantium* and *M. igneus* shared the same RFP, as did *M jannaschii* and *M. formicicum*. Partial *mcrA* gene sequences have been published recently from *Methanoculleus thermophilus* and *Methanosaeta concilii* strain VeAc9 (Lueders *et al*, 2001). The *Taq*I RFP of *M. thermophilus* also matched an OTU detected in one landfill sample. The *Taq*I RFP of *M. concilii* VeAc9 was the same as that of *M. formicicum* and *M. igneus*.

The majority, 53 out of 63 OTUs did not match the *Taq*I RFPs of any of the published sequences that were analysed. To try to identify the methanogen species that gave rise to these OTUs, and to determine the specificity of the OTUs, a number of clones

from each OTU were sequenced. The phylogenetic affiliations of the sequences to *mcrA* sequences from described methanogen species were determined. The results of the sequencing and phylogenetic analysis are described in the next chapter.

3.3.4 Development of DGGE as a rapid measure of diversity

DGGE and TGGE are 'genetic-fingerprinting techniques' that are widely used to investigate microbial diversity in natural ecosystems. The techniques allow the profiling of community complexity, rapidly and without the time-consuming and potentially biased cloning step (Muyzer, 1998; Stackebrandt *et al*, 1998).

Specific limitations of DGGE and TGGE include the underestimation or overestimation of microbial diversity. Estimations of community diversity by DGGE/TGGE are based on the assumption that each band on the gel represents a separate OTU. Diversity may be overestimated due to multiple bands from one organism or PCR-artefacts such as chimeric molecules. Multiple bands can result from the presence of multiple heterogeneous rRNA operons or in the case of the *mcrA* gene from the isogene, *mrtA* (Lueders *et al*, 2001; Stackebrandt *et al*, 1998).

Conversely, diversity may be underestimated because DNA molecules with heterogeneous sequences may exhibit similar melting characteristics, and hence migrate as a single band on the gel (Muyzer, 1998). In addition, the sensitivity of the method used to detect bands on the gels can affect the estimate of diversity (Moeseneder *et al*, 1999; Muyzer *et al*, 1998). Rare sequences may be missed because the bands are too faint to be detected, or very bright bands may obscure fainter bands with a similar mobility.

In this study, the fluorescent dye, SYBR Green I, was used in addition to ethidium bromide. SYBR Green I staining is more sensitive than ethidium bromide staining (Muyzer *et al,* 1998). The bands on gels stained with SYBR Green I were brighter and clearer than those stained with ethidium bromide, and there was less background fluorescence. Greater sensitivity can be achieved by the use of fluorescently labelled PCR products combined with laser-induced fluorescence detection. As little as 1fg of double stranded DNA can be detected (Moeseneder *et al*, 1999).

Most DGGE based investigations of microbial diversity have used 16S rDNA (Casamayor *et al*, 2000; Ovreas *et al*, 1997; Roling *et al*, 2000; Wise *et al*, 1999; Zoetendal *et al*, 1998). DGGE of a functional gene, such as the *mcrA* gene might give better resolution of closely related species compared to DGGE with 16S rDNA. This is because the level of sequence variation in the *mcrA* gene, between pairs of methanogen species is approximately three times greater than that in the 16S rRNA gene (Springer *et al*, 1995). The *mcrA* gene fragments amplified in this study are the ideal size for DGGE. The maximum size of DNA fragments that can be successfully resolved by DGGE is 500bp (Muyzer, 1998). Whereas, DNA fragments significantly less than 500bp might not contain sufficient sequence heterogeneity.

Resolution of the *mcrA* PCR products, sufficient for profiling of the diversity of *mcrA* sequences in landfill samples, was not achieved. However, the results from optimisation of the DGGE and TTGE showed that the techniques have the potential to be useful tools for monitoring methanogen communities in landfill and other environments. Resolution of the *mcrA* PCR products could be improved by further

optimisation of the denaturing gradient conditions, the electrophoresis conditions, and possibly by attachment of the GC-clamp to the *mcrA* forward primer. Muyzer *et al* (1993) found that increased stability of PCR products in DGGE was only observed when a GC-clamp was attached to the 5' primer and not the 3' primer.

3.3.5 Summary

- Excavated refuse and leachate samples were collected from five landfill sites.
- DNA was extracted directly from leachate samples, and by both direct and indirect methods from excavated refuse samples.
- A ~500bp fragment of the *mcrA* gene was successfully amplified by PCR from DNA extracted from all the landfill samples.
- PCR-RFLP screening of clone libraries generated from the *mcrA* PCR products appeared to indicate a greater level of diversity in the landfill methanogen population than had been described by previous studies.
- The diversity and composition of the methanogen population in each landfill sample appeared to be unique.
- The PCR-RFLP analysis appeared to indicate the presence in landfill of methanogen species that had not previously been detected in landfill, in addition to species that had been detected previously in landfill.
- Some resolution of *mcrA* PCR products was achieved with DGGE and TTGE. However, the resolution was not sufficient to allow analysis of complex mixtures of *mcrA* PCR products.

4 Determining the genetic diversity of landfill methanogens by DNA sequencing and phylogenetic analysis

4.1 INTRODUCTION

Genetic fingerprinting techniques such as PCR-RFLP and DGGE allow us an insight into the diversity and structure of microbial communities without cultivation. However, these techniques cannot identify the majority of microorganisms in the environment. To do this, it is common practice to sequence the gene or genes under investigation and compare these sequences with sequences from previously described species. The 16S rRNA gene is by far the most frequently analysed gene for this purpose, and 16S rRNA gene sequences are known from a large number of cultured and described microorganisms. However, it is recognised that the cultured species of *Bacteria* and *Archaea* represent only a minor fraction of the existing diversity (Amann *et al*, 1995). Therefore, it is unlikely that a sequence retrieved from the environment will match exactly the sequence of a described species.

Phylogenetic analysis of sequence data allows the relationship to be inferred, between unknown environmental sequences, and between these sequences and sequences from described species. These phylogenetic affiliations can be used to infer some properties of the unknown organisms in the environment. All the representatives of a particular phylogenetic group may be expected to have the properties that occur commonly in the group (Hugenholtz and Pace, 1996). The inferred properties of novel environmental organisms may be used to predict their function in the

ecosystem, or to predict their nutritional requirements and so guide cultivation attempts.

This chapter describes the sequencing and phylogenetic analysis of *mcrA* clones representing each of the operational taxonomic units (OTUs) identified by the PCR-RFLP analysis of landfill samples (chapter 3).

4.2 RESULTS

Cloned *mcrA* PCR products were selected from each OTU identified by the PCR-RFLP analysis (chapter 3). The DNA sequences were obtained by automated sequencing, as described in section 2.10, and the amino acid sequences were predicted from these DNA sequences. The amino acid sequences were aligned and subject to phylogenetic analysis using programmes from the PHYLIP (Phylogeny Inference Package) suite of programmes, Version 3.57c (Felsenstein, 1995), as described in section 2.12. Alignments of nucleotide and predicted amino acid sequences, from the *mcrA* PCR products cloned from DNA extracted from landfill samples, are presented in appendices A and B. Three methods were used for constructing phylogenetic trees from alignments of *mcrA* amino acid sequences, as described in section 2.12. Each of the methods uses a different algorithm to construct a phylogeny. By comparing the topologies of the trees produced by each method, conclusions could be drawn about the strength or weakness of the phylogeny.

4.2.1 *McrA* sequences from described species of methanogenic *Archaea*

The amino acid sequences of methyl CoM reductase from 34 described species of methanogens were obtained from GenBank (Table 4.1), and included in the analyses to establish the phylogenetic affiliations of the cloned *mcrA* sequences to known methanogens. In addition, partial *mcrA* sequences from 12 methanogen species were determined in this study (Table 4.1), as described in section 2.11. This was done to attempt to identify clusters of sequences from landfill that did not show close affiliation to published *mcrA* sequences from described species. The 12 species were: *Methanocorpusculum aggregans, Methanocorpusculum bavaricum, Methanocorpusculum parvum, Methanoculleus bourgensis* and *Methanospirillum hungatei* from the order *Methanomicrobiales*; *Methanobacterium formicicum, Methanobrevibacter arboriphilicus* and *Methanobrevibacter ruminantium* from the order *Methanobacteriales*; *Methanosaeta concilii, Methanosarcina mazei* and *Methanohalophilus halophilus* from the *Methanosarcinales*; and *Methanopyrus kandleri*, the single species identified in the order *Methanopyrales*. The aligned nucleotide and predicted amino acid sequences of the 12 species are shown in Appendices C and D. In addition, partial *mcrA* gene sequences for three species of *Methanococcus* were provided by P. Riley (personal communication) (Table 4.1).

The phylogenetic positioning of the *mcrA/mrtA* sequences from 49 described methanogen species, including the 12 sequences determined in this study, are shown in Figures 4.1 – 4.3. These analyses clearly showed that the phylogeny constructed from *mcrA* sequences was consistent with that based on 16S rDNA, as shown in Figure 1.8. The five orders of methanogenic *Archaea*, as defined by Boone *et al* (1993), formed distinct clusters, as marked on Figures 4.1 – 4.3 and 1.8. The

Table 4.1 Source organisms for *mcrA* sequences used in phylogenetic analyses and their corresponding *Taq*I restriction fragment sizes.

Taxon	Accession no.[a]	Sizes of DNA fragments (bp)[b]		OTU[c]
		Total	Cut with *Taq*I	
Methanobacterium bryantii [e]	AF313806	467[w]	467[w]	
Methanobacterium bryantii (*mrtA* [d]) [f]	DSM 863	467	53, 414	
Methanobacterium formicicum [f]	DSM 1312	467	9, 21, 170, 267	C
Methanobacterium formicicum (*mrtA* [d]) [g]	DSM 1312	464	464	D
Methanobrevibacter arboriphilicus [g]	DSM 1125	467	86, 381	
Methanobrevibacter ruminantium [g]	DSM 1093	470	470	BE
Methanothermobacter marburgensis [h]	X07794	467	53, 147, 267	
Methanothermobacter thermoautotrophicus [i]	U10036	467	53, 147, 267	
Methanothermobacter thermoautotrophicus (*mrtA* [d]) [j]	AE000883	467	53, 414	
Methanothermobacter wolfei [f]	DSM 2970	467	53, 147, 267	
Methanosphaera stadtmanae [f]	DSM 3091	467	467	
Methanothermus fervidus [k]	J03375	470	128, 342	
Methanothermus fervidus (*mrtA* [d]) [l]	X70765	467	467	
Methanococcus jannaschii [m]	DSM 2661	464	464	D
Methanococcus jannaschii (*mrtA* [d]) [n]	U67465	467	467	
Methanococcus igneus [m]	DSM 5666	470	470	BE
Methanococcus thermolithotrophicus [m]	DSM 2095	467	467	
Methanococcus vannielii [o]	M16893	467	467	
Methanococcus voltae [p]	X07793	467	467	
Methanomicrobium mobile [f]	DSM 1539	491	154, 337	
Methanocorpusculum parvum [g]	DSM 3823	491	104, 185, 202	H
Methanocorpusculum aggregans [g]	DSM 3027	491	104, 185, 202	H
Methanocorpusculum bavaricum [g]	DSM 4179	491	104, 387	G
Methanoculleus bourgensis [g]	DSM 3045	491	51, 53, 154, 233	X
Methanoculleus thermophilus [e]	AF313804	491[w]	51, 53, 179, 208[w]	W
Methanofollis liminatans [f]	DSM 4140	491	51, 53, 387	A
Methanospirillum hungatei [g]	DSM 864	491	51, 53, 387	A
Methanococcoides burtonii [q]	U22234	488	30, 53, 105, 138, 162	
Methanococcoides methylutens [q]	U22235	488	30, 105, 162, 191	
Methanohalobium evestigatum [q]	U22236	488	105, 383	
Methanohalophilus halophilus [q]	DSM 3094	488	69, 105, 122, 192	
Methanohalophilus mahii [q]	U22237	488	69, 105, 122, 192	
Methanohalophilus oregonense [q]	U22242	488	488	
Methanohalophilus portucalensis [q]	U22239	488	69, 105, 122, 192	
Methanohalophilus zhilinae [q]	U22252	488	488	
Methanolobus bombayensis [q]	U22257	488	75, 146, 267	
Methanolobus taylorii [q]	U22243	488	221, 267	
Methanolobus tindarius [q]	U22244	488	146, 342	
Methanolobus vulcani [q]	U22245	488	75, 146, 267	
Methanosarcina acetivorans [q]	U22247	488	45, 53, 93, 105, 192	AD
Methanosarcina barkeri [r]	Y00158	488	45, 53, 93, 105, 192	AD
Methanosarcina mazei [q]	DSM 2053	488	45, 53, 93, 105, 192	AD
Methanosarcina mazei strain C16 [q]	U22258	488	45, 53, 93, 105, 192	AD
Methanosarcina siciliae [q]	U22248	488	45, 53, 93, 105, 192	AD
Methanosarcina thermophila [q]	U22250	488	45, 53, 93, 297	F
Methanosarcina vacuolata [q]	U22251	488	53, 138, 297	
Methanosaeta concilii [s]	DSM 3671	470	128, 342	
Methanosaeta concilii VeAc9 [e]	AF313803	470	470	BE
Methanopyrus kandleri [s]	DSM 6324	467	9, 39, 77, 342	

Table 4.1 continued

Taxon	Accession no.[a]	Sizes of DNA fragments (bp)[b]		OTU[c]
		Total	Cut with *TaqI*	
Unidentified *Methanomicrobiaceae* strain EBac [q]	U22253	488	51, 53, 154, 230	
Unidentified *Methanobacteriales* symbiont of *Reticulitermes speratus* [t]	D64032	470[w]	53, 417[w]	E
Uncultured methanogen ODP8-ME1 [u]	AF121099	467	151, 316	
Uncultured methanogen MRE-ME3 [e]	AF313889	467[w]	170, 297[w]	
Uncultured methanogen RS-MCR04 [e]	AF313810	470	53, 417	E
Uncultured methanogen RS-MCR06 [e]	AF313812	470	53, 417	E
Uncultured methanogen RS-MCR07 [e]	AF313813	467	179, 288	
Uncultured methanogen RS-MCR10 [e]	AF313816	467	30, 42, 53, 65, 117, 160	
Uncultured methanogen RS-MCR12 [e]	AF313818	464	53, 411	
Uncultured methanogen RS-MCR25 [e]	AF313829	467	53, 112, 117, 185	
Uncultured methanogen RS-MCR29 [e]	AF313833	464	53, 411	
Uncultured methanogen RS-MCR36 [e]	AF313839	488	45, 53, 93, 297	F
Uncultured methanogen RS-MCR38 [e]	AF313841	470	39, 203, 228	
Uncultured methanogen RS-MCR40 [e]	AF313843	467	170, 297	
Uncultured methanogen RS-MCR45 [e]	AF313848	467	179, 288	
Uncultured methanogen RS-ME42 [e]	AF313875	488[w]	24, 53, 69, 342[w]	
Uncultured methanogen RS-ME43 [e]	AF313876	488[w]	53, 138, 297[w]	
Uncultured archaeon 20B [v]	AF268631	470[w]	53, 417[w]	E
Uncultured archaeon 20D [v]	AF268629	470	128, 342	
Uncultured archaeon 26A [v]	AF268624	488[w]	45, 53, 93, 105, 192[w]	AD
Uncultured archaeon 27D [v]	AF268626	467	53, 414	
Uncultured archaeon 80B [v]	AF268652	491[w]	51, 53, 387[w]	A
Uncultured archaeon 82D [v]	AF268656	491	30, 51, 53, 154, 203	T
Uncultured archaeon 83D [v]	AF268653	464	464	D
Uncultured archaeon 85A [v]	AF268654	473[w]	39, 77, 105, 252[w]	
Uncultured archaeon 90C [v]	AF268651	491[w]	51, 53, 65, 120, 202[w]	
Uncultured archaeon 91B [v]	AF268647	491[w]	30, 51, 53, 154, 203[w]	T
Uncultured archaeon 94D [v]	AF268650	488[w]	45, 105, 146, 192[w]	
Uncultured archaeon 95A [v]	AF268648	464[w]	464[w]	D

Key: [a] GenBank accession numbers for the complete or partial *mcrA* nucleotide sequence, except those numbers prefixed with DSM, which are the accession numbers for strains in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

[b] DNA sequences of the *mcrA* gene were used to determine the size in base pairs of the restriction fragments that would be obtained if *mcrA* PCR products were cut with the tetrameric restriction endonucleases, *Taq*I.

[c] Operational taxonomic unit as determined by *Taq*I RFLP, refer to Table 3.3.

[d] All sequences were *mcrA* except for those marked as *mrtA*.

Sequences used in this study were determined by: [e] Lueders *et al* (2001) [f] Luton (1996), [g] this study, [h] Bokranz *et al* (1988), [i] Pihl *et al* (1994), [j] Smith *et al* (1997), [k] Weil *et al* (1988), [l] Lehmacher and Klenk (1994), [m] P. Riley (personal communication), [n] Bult *et al* (1996), [o] Cram *et al* (1987), [p] Klein *et al* (1988), [q] Springer *et al* (1995), [r] Bokranz & Klein (1987), [s] Nolling *et al* (1996), [t] Ohkuma *et al* (1995) [u] Bidle *et al* (1999) and [v] Hougaard and Westermann (2000).

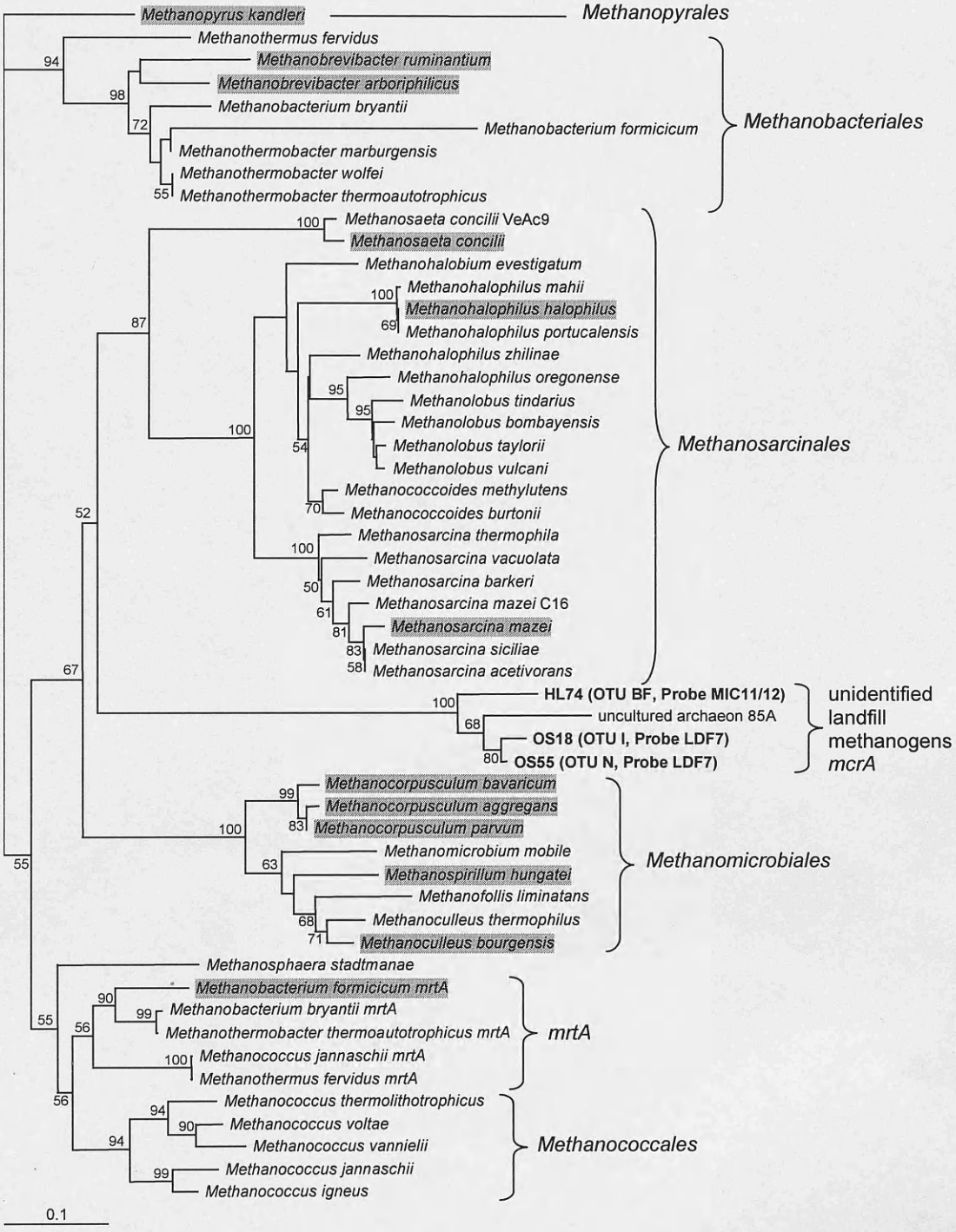
[w] Fragment sizes estimated from alignments because the sequences were not complete.

topology of the clusters of each of the methanogen orders was similar in the 16S rDNA tree and the three *mcrA* trees.

The sequence amplified from *M. formicicum*, in this study, formed a distinct cluster with four sequences of *mrtA*, obtained from GenBank, (Figures 4.1 – 4.3). A second sequence amplified from the same strain of *M. formicicum* by Luton (1996) formed a cluster with the *mcrA* sequences from other members of the order *Methanobacteriales*. The amplification of *mrtA*, an isogene of *mcrA*, using primers designed to amplify *mcrA* has been documented by Lueders *et al* (2001). Phylogenetically, the sequences of *mrtA* are distinct from *mcrA* (Lueders *et al*, 2001; Springer *et al*, 1995). The three methods of tree construction showed the cluster of *mrtA* sequences to be consistently most closely related to the cluster of *mcrA* sequences from the *Methanococcales*. In addition to *M. formicicum*, PCR products were amplified from two other species of *Methanobacterium*, *M. bryantii* and *M. espanolae*. However, direct sequencing of these PCR products consistently gave poor results. It seems likely that this was because both the *mcrA* and *mrtA* genes had been amplified from these species. The sequences of *mcrA* and *mrtA* from *M bryantii* were determined separately by Luton (1996) and Lueders *et al* (2001). Phylogenetic analysis of these sequences in this study showed that one was affiliated with *mcrA* from *Methanobacteriales* and the other with *mrtA* from *Methanobacteriales* (Figures 4.1 – 4.3).

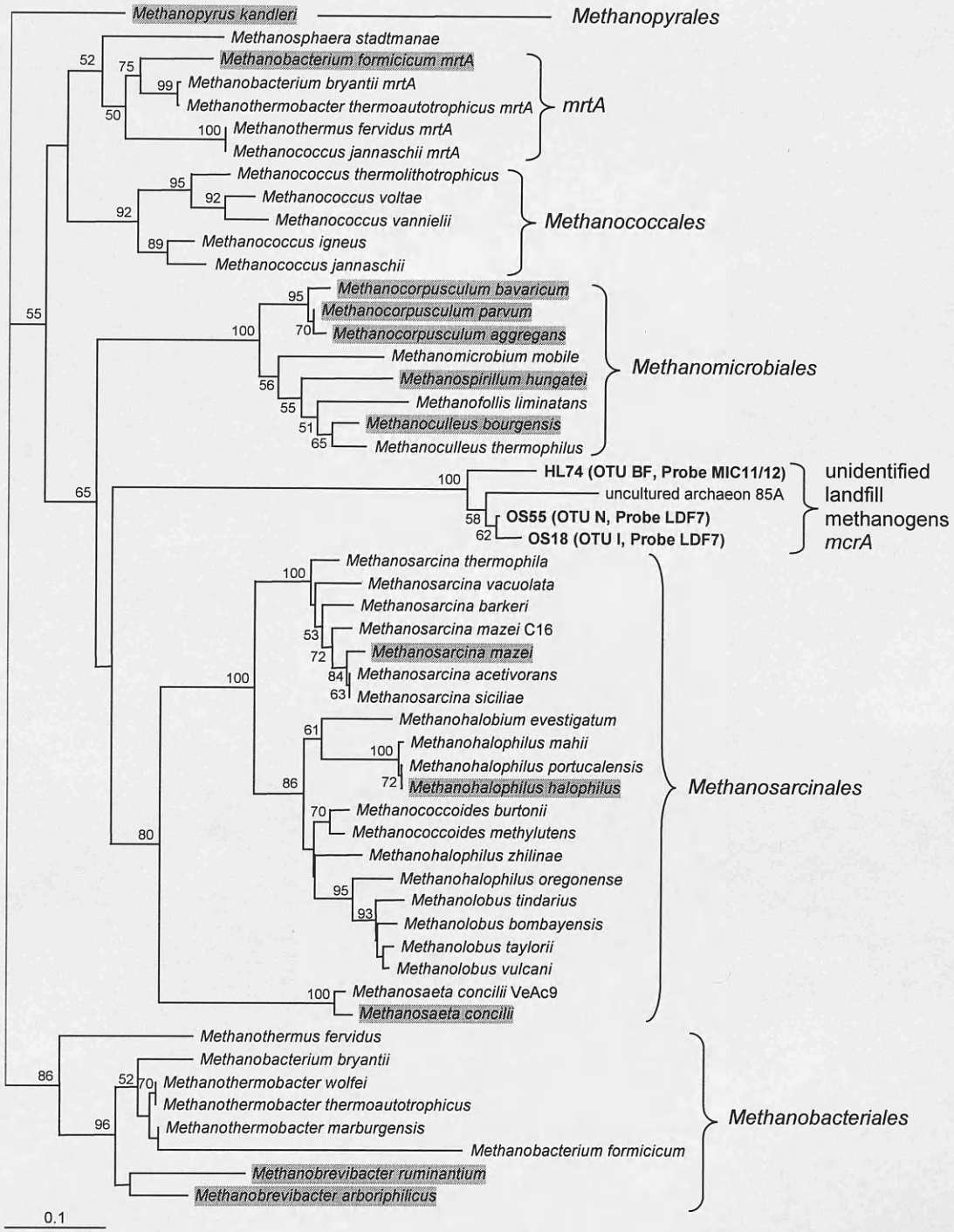
The phylogenetic positioning of the other *mcrA* sequences from described species, determined in this study was also consistent with their taxonomy (Table 1.5) and the 16S rDNA based phylogeny (Figure 1.8). *McrA* sequences from two species of the

Figure 4.1 Fitch distance-matrix tree indicating the relationships of 49 described species of methanogenic *Archaea*, and a cluster of unidentified *mcrA* sequences amplified from landfill.



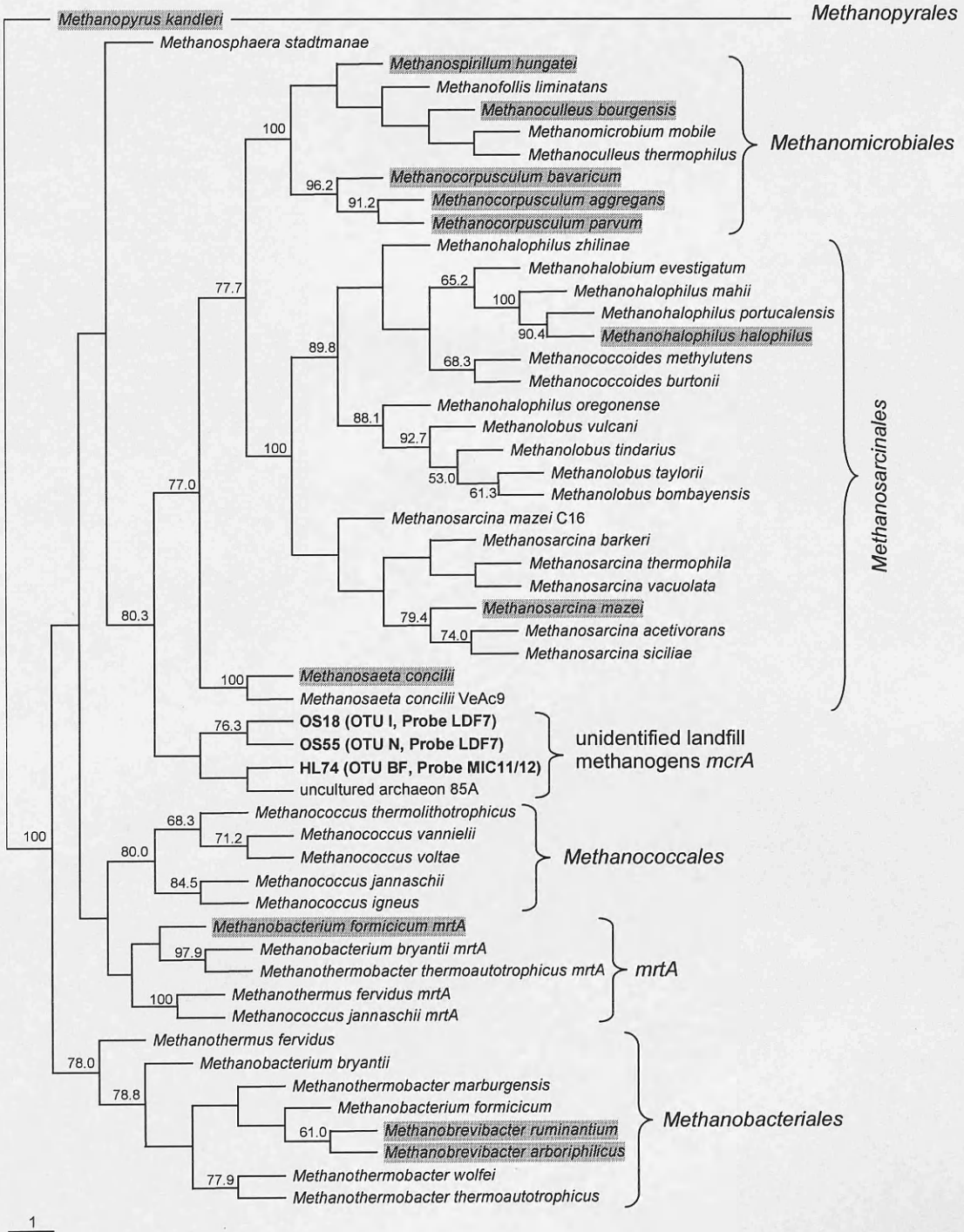
Legend: The phylogenetic tree was constructed from predicted *mcrA* amino acid sequences (146 positions) using Fitch distance matrix analysis, as described in section 2.12. The sources of the *mcrA* sequences are listed in Table 4.1. Sequences of described species determined in this study are shaded. *Methanopyrus kandleri* was used as the outgroup. The bootstrap values above 50% are shown at nodes. HL and OS indicate cloned *mcrA* PCR products from the Hermitage leachate and Odcombe excavated refuse samples respectively (bold face). The OTUs as determined by *Taq*I RFLP (see Table 3.3), and the *mcrA* probes hybridising to the landfill sequences, are indicated after each clone.

Figure 4.2 Neighbor-joining tree indicating the relationships of 49 described species of methanogenic *Archaea*, and a cluster of unidentified *mcrA* sequences amplified from landfill.



Legend: The phylogenetic tree was constructed from predicted *mcrA* amino acid sequences (146 positions) using neighbor-joining analysis, as described in section 2.12. The sources of the *mcrA* sequences are listed in Table 4.1. Sequences of described species determined in this study are shaded. *Methanopyrus kandleri* was used as the outgroup. The bootstrap values above 50% are shown at nodes. HL and OS indicate cloned *mcrA* PCR products from the Hermitage leachate and Odcombe excavated refuse samples respectively (bold face). The OTUs as determined by *Taq*I RFLP (see Table 3.3), and the *mcrA* probes hybridising to the landfill sequences, are indicated after each clone.

Figure 4.3 Parsimony tree indicating the relationships of 49 described species of methanogenic *Archaea*, and a cluster of unidentified *mcrA* sequences amplified from landfill.



Legend: The phylogenetic tree was constructed from predicted *mcrA* amino acid sequences (146 positions) using parsimony analysis, as described in section 2.12. The sources of the *mcrA* sequences are listed in Table 4.1. Sequences of described species determined in this study are shaded. *Methanopyrus kandleri* was used as the outgroup. The bootstrap values above 50% are shown at nodes. HL and OS indicate cloned *mcrA* PCR products from the Hermitage leachate and Odcombe excavated refuse samples respectively (bold face). The OTUs as determined by *Taq*I RFLP (see Table 3.3), and the *mcrA* probes hybridising to the landfill sequences, are indicated after each clone.

genus *Methanobrevibacter*, *M. arboriphilicus* and *M. ruminantium*, were determined. Phylogenetic analyses showed these sequences to be most closely related to each other, and to group within the phylogenetic radiation of the *Methanobacteriales mcrA* sequences, in all three trees (Figures 4.1 – 4.3).

Sequences amplified from *M. aggregans*, *M. bavaricum* and *M. parvum* formed a tight cluster supported by high bootstrap values in all three trees. This cluster diverged from a cluster containing five sequences from other members of the *Methanomicrobiales*. In all three trees, this node was supported by a bootstrap value of 100. The divergence of the three *Methanocorpusculum* species into a separate cluster within the *Methanomicrobiales* is consistent with their classification as a separate family, *Methanocorpusculaceae*. The sequence amplified from *Methanoculleus bourgensis* showed closest affiliation to the *mcrA* sequence from *Methanoculleus thermophilus* using the Fitch distance-matrix method (Figure 4.1) and the Neighbor-Joining method (Figure 4.2), but not using the Parsimony method (Figure 4.3). The topology of the cluster containing the *Methanoculleus* sequences was supported by bootstrap values greater than 50% in the Fitch distance-matrix tree and the Neighbor-Joining tree, but not in the Parsimony tree. The sequence amplified from *M. hungatei* grouped with sequences from *M. mobile*, *M. liminatans*, *M. bourgensis* and *M. thermophilus*. The last four species are members of the family *Methanomicrobiaceae*, while Boone *et al* (1993) proposed that *M. hungatei* be moved to a separate family, *Methanospirillaceae*.

Sequences of *mcrA* were obtained from *M. concilii*, *M. halophilus* and *M. mazei*, all members of the *Methanosarcinales*. On the Fitch distance-matrix and Neighbor-

Joining trees, the sequence of *M. concilii* (DSM 3671) formed a deep-branching cluster on the *Methanosarcinales* branch, with the *mcrA* sequence from *M. concilii* strain VeAc9. On the Parsimony tree, the *Methanosaeta* cluster split from the other *Methanosarcinales* before the divergence of the *Methanosarcinales* and *Methanomicrobiales*. On all three trees, the nodes at which these three groups diverged were supported by bootstrap values greater than 50%. The sequence obtained from *M. halophilus* (DSM 3094) was identical to that determined by Springer *et al* (1995). The *mcrA* sequence determined for *M. mazei* (DSM 2053) differed from that determined for *M. mazei* strain S-6 (OCM 26) (Springer *et al*, 1995) at three nucleotides, which resulted in three differences in the amino acid sequences.

Having confirmed that the phylogeny based on the partial *mcrA* sequences was in agreement with the phylogeny based on 16S rDNA, the relationships of the sequences amplified from landfill to described species could be established.

4.2.2 The phylogeny of *mcrA* sequences from landfill

The DNA sequence was determined for 147 cloned *mcrA/mrtA* PCR products amplified from six landfill samples. The phylogenetic affiliations of all of these sequences were determined. For clarity, the phylogenetic affiliations of just 90 out of the 147 cloned *mcrA/mrtA* PCR products are shown in Figures 4.1 – 4.12. The clones were selected so that, as far as possible, each RFLP was represented by at least two clones, or one from each landfill sample in which the RFLP was detected. To make the presentation of the phylogenetic trees clearer, the sequences were split into three groups based on their phylogenetic affiliation to sequences within the orders *Methanobacteriales, Methanomicrobiales* and *Methanosarcinales*. Phylogenetic trees

were then constructed from the three groups of sequences using the Fitch distance-matrix, Neighbor-Joining and Parsimony methods (Figures 4.4 – 4.12). A cluster of three clones, named "unidentified landfill methanogens *mcrA*", did not show close affiliation to any of the orders. This cluster is therefore shown in Figures 4.1 – 4.3.

The majority of *mcrA* sequences in the GenBank database (Benson *et al*, 2000) are from uncultured methanogens or *Archaea*, detected in rice field soil or anaerobic digesters (Hougaard and Westermann, 2000; Lueders *et al*, 2001). To determine if any *mcrA* sequences from landfill were closely affiliated to these sequences from uncultured methanogens, the standard BLAST (Altschul *et al*, 1990) was used to search GenBank for the closest matches to the amino acid sequences of these clones (Table 4.2). Twenty-nine clones were selected to represent each of the clusters identified by the phylogenetic analyses to use as query sequences. The closest matches to 25 out of the 29 clones were *mcrA* or *mrtA* sequences from uncultured methanogens or *Archaea*. Twenty-nine sequences from uncultured or incompletely described methanogens (Table 4.1) were included in the phylogenetic analyses (Figures 4.4 – 4.12).

4.2.2.1 Novel methanogenic lineage in landfill

Two clones amplified from the Odcombe landfill sample, OS18 and OS55, and one clone from the Hermitage landfill leachate sample, HL74, formed a deep-branching cluster. This cluster appeared distinct from, but related to, the methanogenic orders *Methanosarcinales* and *Methanomicrobiales* (Figures 4.1 – 4.3). The Fitch distance-matrix method and Neighbor-Joining method both showed this cluster splitting from

Table 4.2 Sequence identity values between cloned sequences amplified from landfill and their closest matches identified by BLAST search of the GenBank database.

Clone	Closest match	Sequence identity
HL74	uncultured archaeon 85A (AF268654)	86% (113/131)
BSS12	*Methanothermus fervidus mrtA* (X70765)	88% (122/138)
MS51	uncultured archaeon 83D (AF268653)	93% (128/137)
BSS26	uncultured archaeon 83D (AF268653)	94% (129/137)
OS37	uncultured archaeon 83D (AF268653)	94% (130/137)
OS70	uncultured archaeon 83D (AF268653)	94% (130/137)
OS82	uncultured methanogen RS-MCR45 (AF313848)	91% (126/138)
OS105	uncultured methanogen RS-MCR07 (AF313813)	95% (132/138)
BSS54	uncultured methanogen RS-MCR07 (AF313813)	96% (133/138)
BSS2	uncultured archaeon 27D (AF268626)	92% (127/138)
MS6	uncultured archaeon 20B (AF268631)	93% (125/134)
MS26	uncultured archaeon 20B (AF268631)	93% (125/134)
BSS59	uncultured methanogen MRE-MCR6 (AF313887)	74% (104/139)
BSS43	uncultured archaeon 82D (AF268656)	86% (126/146)
HL110	uncultured archaeon 90C (AF268651)	85% (119/140)
PL126	uncultured archaeon 90C (AF268651)	82% (116/140)
BSD63	*Methanospirillum hungatei* (AF313805)	89% (129/144)
MS37	*Methanospirillum hungatei* (AF313805)	93% (135/144)
OS77	uncultured archaeon 82D (AF268656)	81% (119/146)
PL21	uncultured archaeon 82D (AF268656)	80% (117/146)
BSS8	uncultured archaeon 80B (AF268652)	75% (107/142)
BSS21	uncultured methanogen RS-MCR02 (AF313808)	97% (141/145)
BSS50	uncultured archaeon 26A (AF268624)	98% (131/133)
BSS65	*Methanosarcina mazei* C16 (U22258)	100% (145/145)
MS22	uncultured methanogen RS-ME43 (AF313876)	97% (140/143)
MS42	uncultured methanogen RS-MCR36 (AF313839)	95% (139/145)
PL53	uncultured archaeon 20D (AF268629)	86% (120/139)
BSD43	uncultured methanogen RS-MCR38 (AF313841)	93% (130/139)
BSD79	uncultured methanogen RS-MCR38 (AF313841)	94% (130/139)

Key: Cloned *mcrA* sequences from landfill were selected from each cluster identified by phylogenetic analyses (Figures 4.1 – 4.12). Standard BLAST was used to find the closest matches in the GenBank database, to the predicted amino acid sequences of the landfill clones.

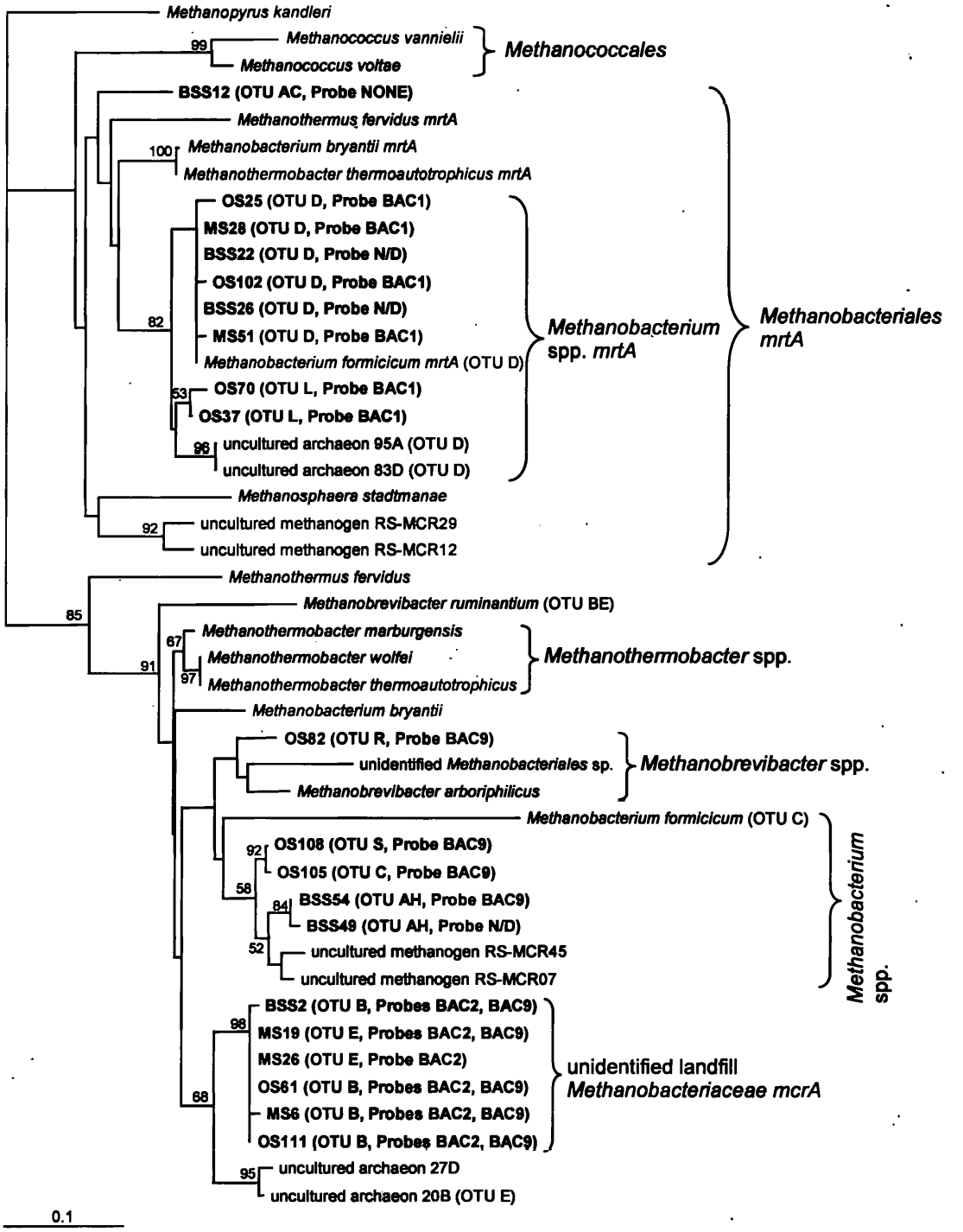
the *Methanosarcinales* after the divergence of the *Methanomicrobiales* (Figures 4.1, 4.2). The Parsimony method showed the cluster diverging before the divergence of the *Methanosarcinales* and *Methanomicrobiales* (Figure 4.3). A sequence from an uncultured archaeon, detected in an anaerobic digester (Hougaard and Westermann, 2000) showed 86% identity with the amino acid sequence of clone HL74. This sequence, uncultured archaeon 85A, was closely affiliated with the three sequences from landfill using the three methods of tree construction. HL74 showed amino acid sequence identities of 55-60% with members of all five orders of methanogens. These low values support the conclusion from the phylogenetic analysis that this cluster of sequences is distinct from any described species of methanogen, and may represent an uncultured novel methanogenic lineage.

4.2.2.2 *Methanobacteriales* in landfill

Sequences showing affiliation to the *Methanobacteriales mcrA* were detected in the Mucking, Odcombe and Brogborough 3m excavated refuse samples, but not in the Brogborough sample taken from a depth of 18m, or in samples from the Poyle and Hermitage landfills. Landfill clones related to *Methanobacteriales mcrA* formed three distinct clusters with the three methods of tree construction (Figures 4.4 – 4.6).

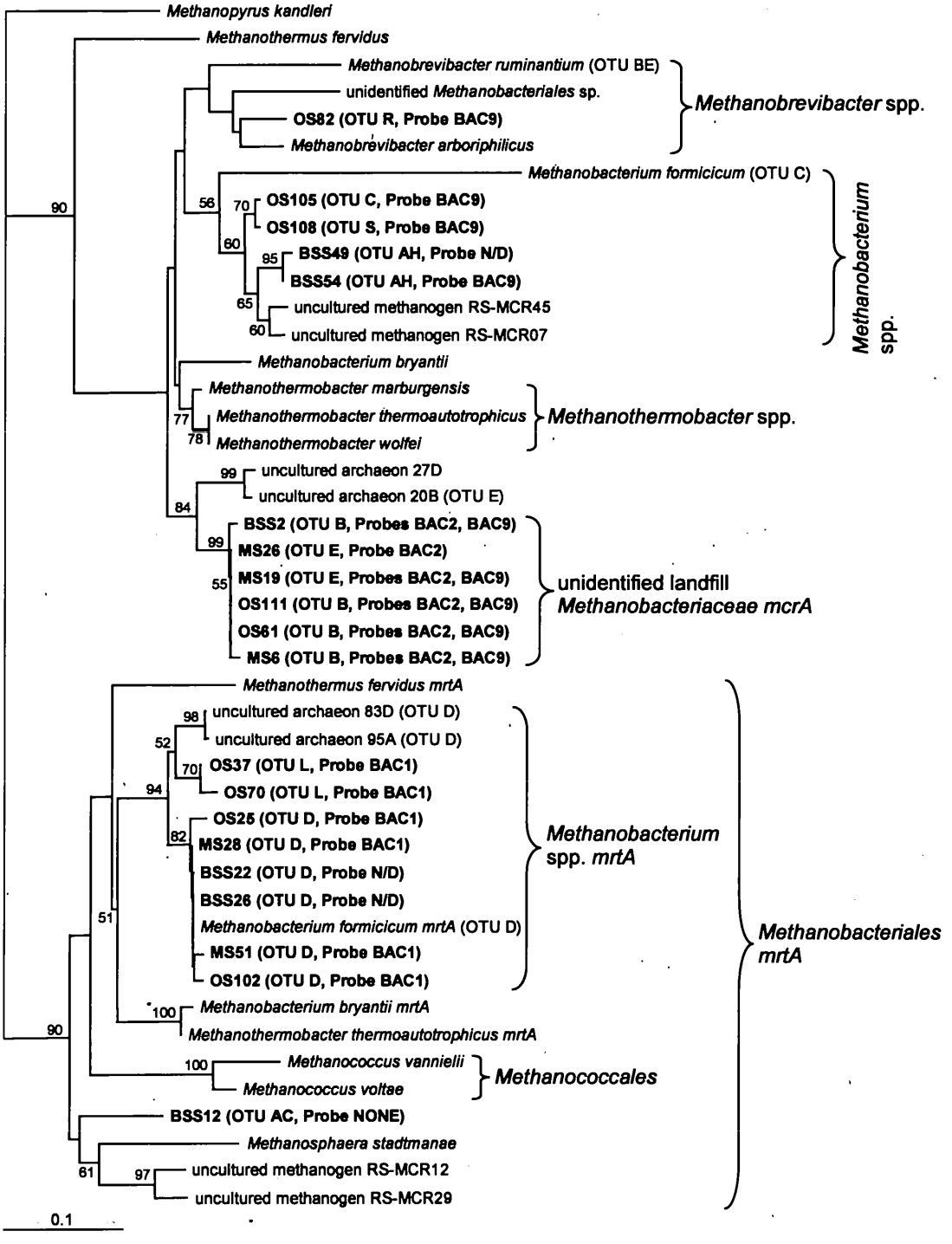
A single clone, OS82, that appeared closely related to *Methanobrevibacter arboriphilicus*, was sequenced from the Odcombe landfill. These two sequences had an amino acid sequence identity of 93%. Two other sequences from *Methanobrevibacter ruminantium* and an unidentified *Methanobacteriales* symbiont of the termite *Reticulitermes speratus*, grouped in the same cluster using the Neighbor-Joining method (Figure 4.5), but not with the other two methods (Figures

Figure 4.4 Fitch distance-matrix tree of cloned *mcrA* PCR products from landfill, showing affiliation with species of the order *Methanobacteriales*.



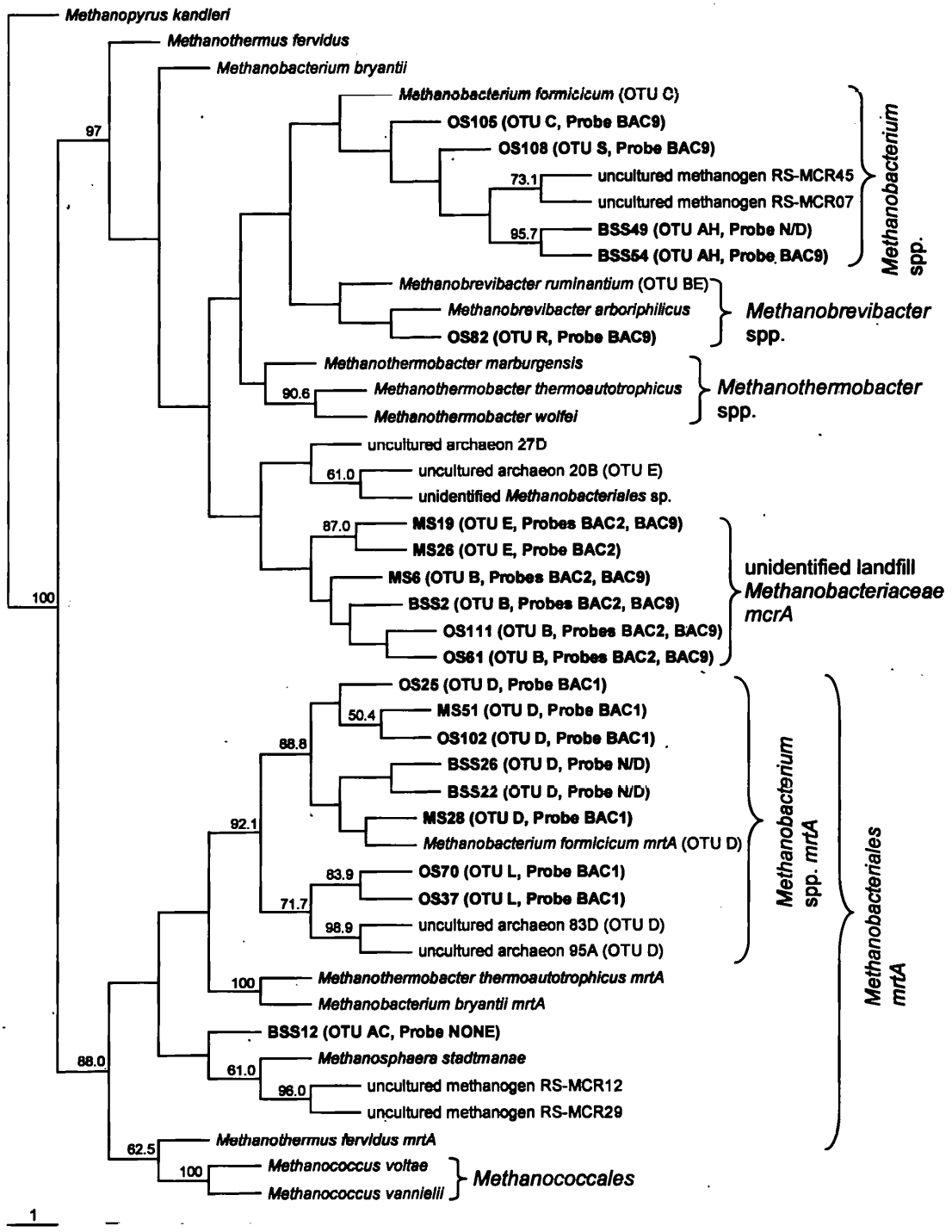
Legend: The phylogenetic tree was constructed from predicted *mcrA* amino acid sequences (146 positions) using Fitch distance-matrix analysis, as described in section 2.12. The sources of the *mcrA* sequences for described species are listed in Table 4.1. *Methanopyrus kandleri* is used as the outgroup. The bootstrap values above 50% are shown at nodes. BSS, MS and OS indicate cloned *mcrA* PCR products from the Brogborough 3m, Mucking and Odcombe excavated refuse samples respectively (bold face). The OTUs as determined by *Taq*I RFLP (see Tables 3.3 and 4.1), and the *mcrA* probes hybridising to the landfill sequences, are indicated after each clone. NONE = not detected by any probe. N/D = not determined.

Figure 4.5 Neighbor-joining tree of cloned *mcrA* PCR products from landfill, showing affiliation with species of the order *Methanobacteriales*.



Legend: The phylogenetic tree was constructed from predicted *mcrA* amino acid sequences (146 positions) using neighbor-joining analysis, as described in section 2.12. The sources of the *mcrA* sequences for described species are listed in Table 4.1. *Methanopyrus kandleri* is used as the outgroup. The bootstrap values above 50% are shown at nodes. BSS, MS and OS indicate cloned *mcrA* PCR products from the Brogborough 3m, Mucking and Odcombe excavated refuse samples respectively (bold face). The OTUs as determined by *Taq*I RFLP (see Tables 3.3 and 4.1), and the *mcrA* probes hybridising to the landfill sequences, are indicated after each clone. NONE = not detected by any probe. N/D = not determined.

Figure 4.6 Parsimony tree of cloned *mcrA* PCR products from landfill, showing affiliation with species of the order *Methanobacteriales*.



Legend: The phylogenetic tree was constructed from predicted *mcrA* amino acid sequences (146 positions) using parsimony analysis, as described in section 2.12. The sources of the *mcrA* sequences for described species are listed in Table 4.1. *Methanopyrus kandleri* is used as the outgroup. The bootstrap values above 50% are shown at nodes. BSS, MS and OS indicate cloned *mcrA* PCR products from the Brogborough 3m, Mucking and Odcombe excavated refuse samples respectively (bold face). The OTUs as determined by *Taq*I RFLP (see Tables 3.3 and 4.1), and the *mcrA* probes hybridising to the landfill sequences, are indicated after each clone. NONE = not detected by any probe. N/D = not determined.

4.4 and 4.6). The cluster containing OS82 and *M. arboriphilicus* was not supported by bootstrap values greater than 50% in any of the three trees.

Sequences from the Odcombe and Brogborough 3m landfill sample (OS108, OS105, BSS54 and BSS49) formed a cluster with *mcrA* sequences of uncultured methanogens from rice field soil (Figures 4.4 – 4.6). Clones were also sequenced from the Mucking sample belonging to this cluster. Lueders *et al* (2001) identified the sequences, RS-MCR07 and RS-MCR45, from rice field soil as belonging to the family *Methanobacteriaceae*, which includes the genera *Methanobacterium, Methanobrevibacter, Methanosphaera* and *Methanothermobacter*. The described species to which this cluster of landfill clones showed closest affiliation was *Methanobacterium formicicum*, with 80% amino acid sequence identity between clone OS105 and *M. formicicum*.

A third cluster of landfill clones grouped within the phylogenetic radiation of the *Methanobacteriaceae mcrA* (Figures 4.4 – 4.6). This cluster included clones from three landfills, Mucking, Odcombe and Brogborough (3m sample). The sequences in this cluster were identical, except for single amino acid differences in clones MS6 and BSS2. This cluster did not show close affiliation to any described methanogen species. The closest matches to these sequences in the GenBank database, identified using BLAST, were four sequences from uncultured *Archaea* detected in anaerobic digesters (Hougaard and Westermann, 2000). Two of these sequences, 20B and 27D, had 94% and 92% amino acid sequence identity, respectively, to the sequences from landfill.

4.2.2.3 *MrtA* sequences amplified from landfill

Sequences were amplified from the Mucking, Odcombe and Brogborough landfill samples that were affiliated with *mrtA* sequences from described methanogen species (Figures 4.4 – 4.6). The *mrtA* sequences from landfill formed three distinct clusters in each of the phylogenetic trees generated by the Fitch distance-matrix, Neighbor-Joining and Parsimony methods (Figures 4.4 – 4.6). The largest cluster contained sequences from the Mucking, Odcombe and Brogborough 3m samples, with only one to two amino acid differences between the sequences. The sequence from *M. formicicum*, determined in this study, was identical to sequences in this cluster. A second cluster contained *mrtA* sequences that were detected only in the Odcombe landfill. These sequences showed closest affiliation to sequences from two uncultured *Archaea* from anaerobic digesters (Hougaard and Westermann, 2000). The sequence from a described methanogen species to which the landfill sequences showed closest affiliation was that of *M. formicicum*. The two landfill clones, OS37 and OS70 had 96% and 95% amino acid sequence identity, respectively to *M. formicicum mrtA*. These two clusters were named together as *Methanobacterium* spp. *mrtA*. A single clone, BSS12, from the Brogborough 3m sample, formed a cluster with a sequence from *Methanosphaera stadtmanae* and two sequences from rice field soil, using the Neighbor-Joining and Parsimony methods, but not the Fitch distance-matrix method (Figures 4.4 – 4.6). BSS12 and *Methanosphaera stadtmanae* had 81% amino acid sequence identity.

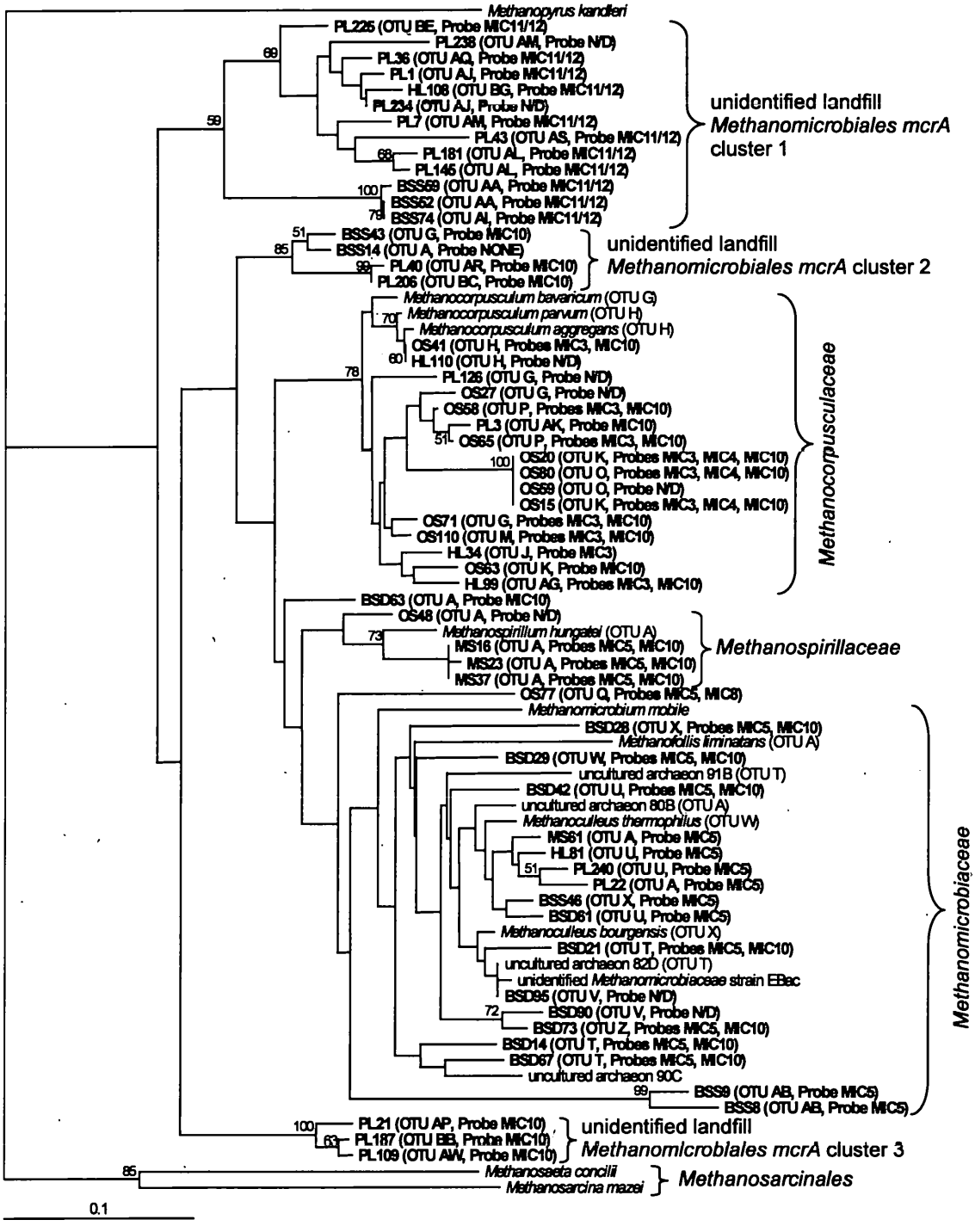
4.2.2.4 *Methanomicrobiales* in landfill

The selection of *mcrA* clones for sequencing was based on obtaining representatives of each OTU identified by the PCR-RFLP analysis (chapter 3). Sixty-one percent of

the 147 clones sequenced grouped within the phylogenetic radiation of the *Methanomicrobiales*. To make the phylogenetic trees easier to interpret, the phylogenetic affiliation of just 59 of the 90 *Methanomicrobiales* sequences is shown in Figures 4.7 to 4.9. As mentioned previously, the clones were selected so that, as far as possible, each RFLP was represented by at least two clones, or one from each landfill sample in which the RFLP was detected. Phylogenetic analyses of the *Methanomicrobiales* sequences revealed six stable clusters of sequences, as indicated in Figures 4.7 to 4.9. Three of these clusters were tentatively identified as *Methanocorpusculaceae, Methanomicrobiaceae* and *Methanospirillaceae*, based on the *mcrA* sequences from described species that grouped within the clusters. Three further clusters of landfill clones did not show affiliation to any described species. These clusters were named "unidentified landfill *Methanomicrobiales mcrA* clusters 1, 2 and 3". In general, the *Methanomicrobiales* trees were not supported by many bootstrap values greater than 50% and the topologies of the trees was slightly different using each method of tree construction. However, the six clusters indicated in the trees were present using all three methods.

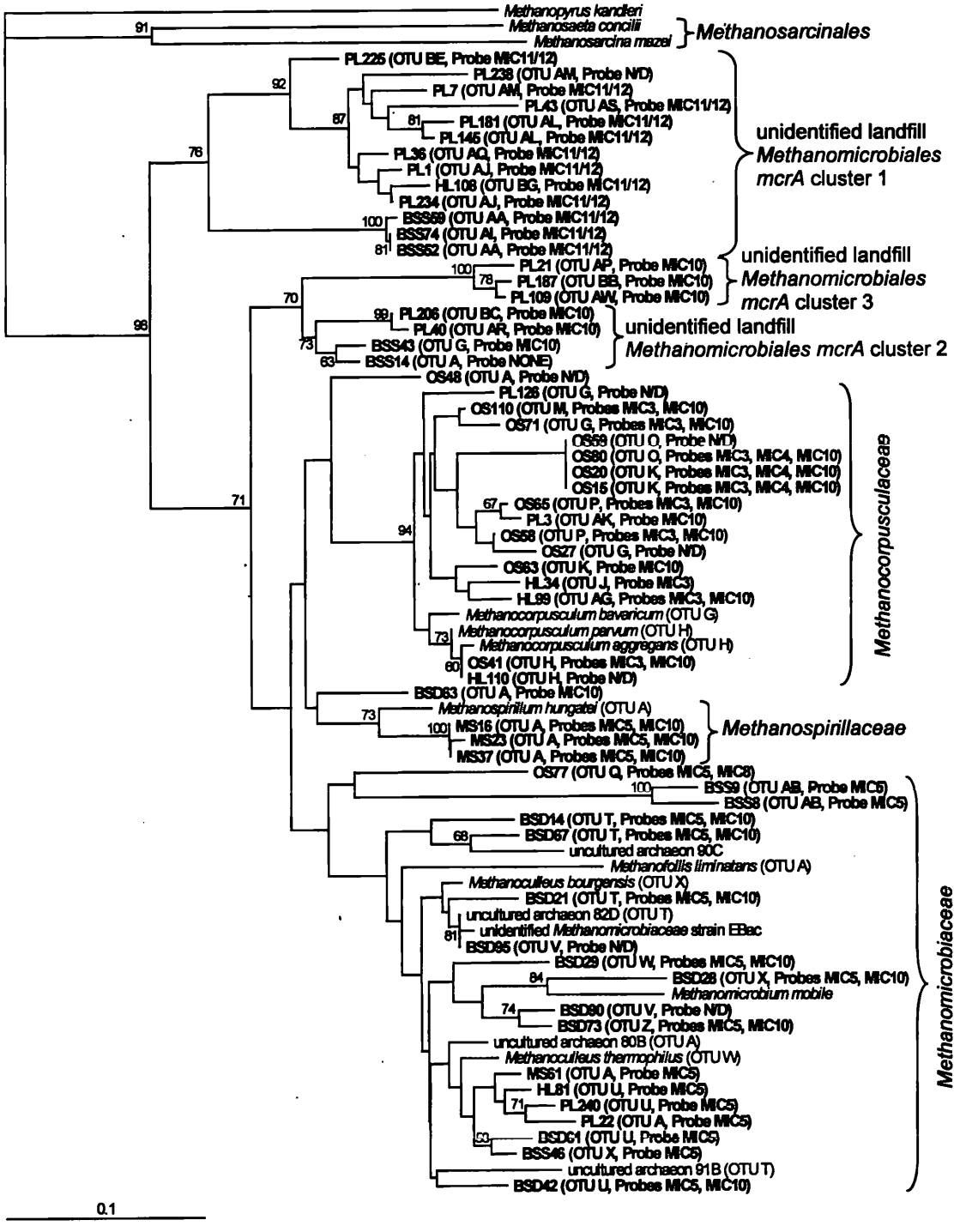
The *Methanocorpusculaceae* cluster diverged into two groups, one of which contained sequences from three species of the genus *Methanocorpusculum*, *M. aggregans, M. bavaricum* and *M. parvum*. This group contained sequences from the Odcombe and Hermitage landfills. The second group contained sequences from the Odcombe, Poyle and Hermitage landfills, but no sequences from described species. This second group may be affiliated with the genus *Methanocalculus*, the only other genus in the family *Methanocorpusculaceae*. This would be in agreement with the phylogeny based on 16S rDNA, as shown in Figure 1.8. A species of

Figure 4.7 Fitch distance-matrix tree of cloned *mcrA* PCR products from landfill that show affiliation with species of the order *Methanomicrobiales*.



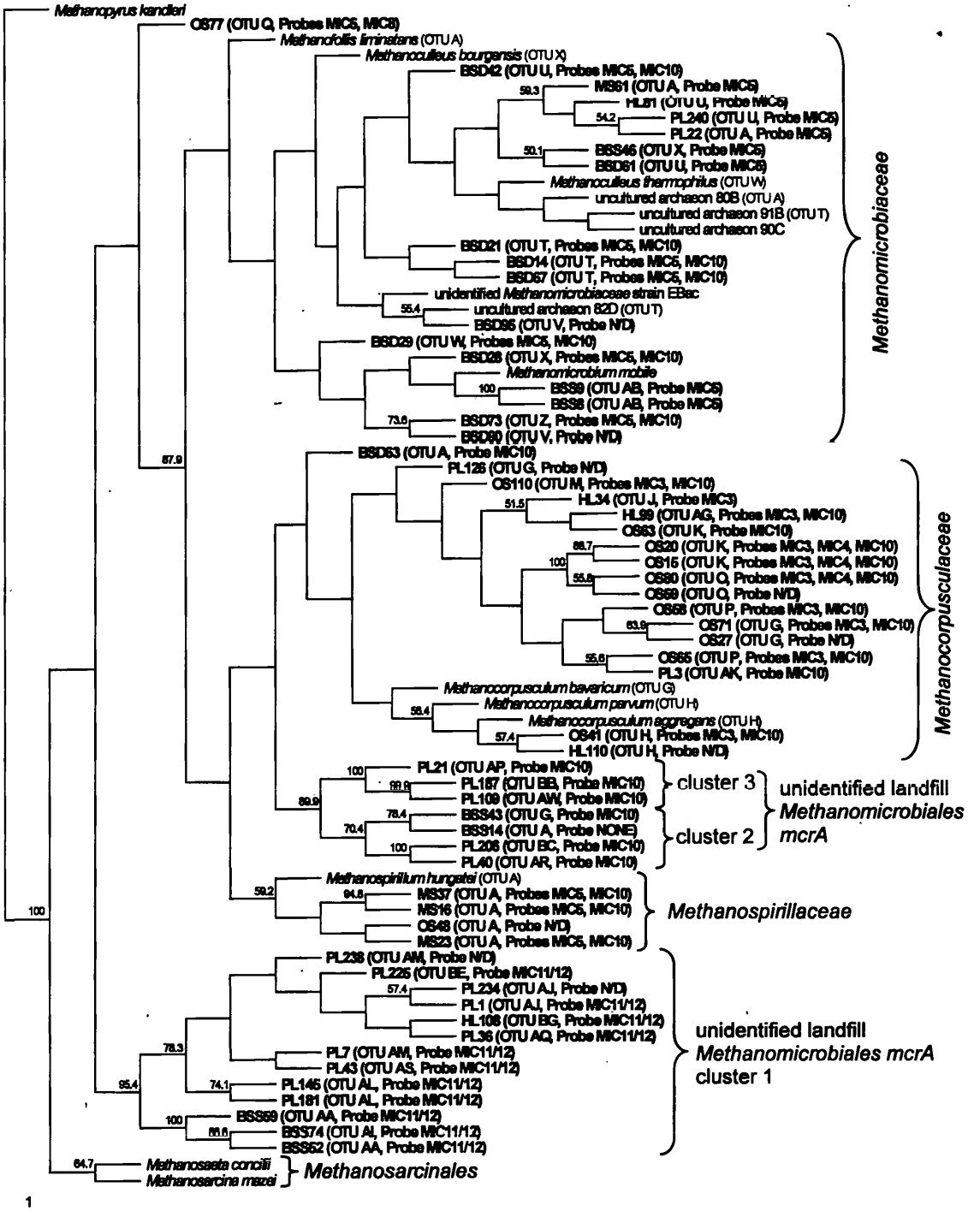
Legend: The phylogenetic tree was constructed from predicted *mcrA* amino acid sequences (146 positions) using Fitch distance-matrix analysis, as described in section 2.12. The sources of the *mcrA* sequences for described species are listed in Table 4.1. *Methanopyrus kandleri* is used as the outgroup. The bootstrap values above 50% are shown at nodes. BSS, BSD, HL, MS, OS and PL indicate cloned *mcrA* PCR products from the Brogborough 3m, Brogborough 18m, Hermitage leachate, Mucking, Odcombe and Poyle landfill samples respectively (bold face). The OTUs as determined by *Taq*I RFLP (see Tables 3.3 and 4.1), and the *mcrA* probes hybridising to the landfill sequences, are indicated after each clone. NONE = not detected by any probe. N/D = not determined.

Figure 4.8 Neighbor-joining tree of cloned *mcrA* PCR products from landfill that show affiliation with species of the order *Methanomicrobiales*.



Legend: The phylogenetic tree was constructed from predicted *mcrA* amino acid sequences (146 positions) using neighbor-joining analysis, as described in section 2.12. The sources of the *mcrA* sequences for described species are listed in Table 4.1. *Methanopyrus kandleri* is used as the outgroup. The bootstrap values above 50% are shown at nodes. BSS, BSD, HL, MS, OS and PL indicate cloned *mcrA* PCR products from the Brogborough 3m, Brogborough 18m, Hermitage leachate, Mucking, Odcombe and Poyle landfill samples respectively (bold face). The OTUs as determined by *Taq*I RFLP (see Tables 3.3 and 4.1), and the *mcrA* probes hybridising to the landfill sequences, are indicated after each clone. NONE = not detected by any probe. N/D = not determined.

Figure 4.9 Parsimony tree of cloned *mcrA* PCR products from landfill that show affiliation with species of the order *Methanomicrobiales*.



Legend: The phylogenetic tree was constructed from predicted *mcrA* amino acid sequences (146 positions) using parsimony analysis, as described in section 2.12. The sources of the *mcrA* sequences for described species are listed in Table 4.1. *Methanopyrus kandleri* is used as the outgroup. The bootstrap values above 50% are shown at nodes. BSS, BSD, HL, MS, OS and PL indicate cloned *mcrA* PCR products from the Brogborough 3m, Brogborough 18m, Hermitage leachate, Mucking, Odcombe and Poyle landfill samples respectively (bold face). The OTUs as determined by *Taq*I RFLP (see Tables 3.3 and 4.1), and the *mcrA* probes hybridising to the landfill sequences, are indicated after each clone. NONE = not detected by any probe. N/D = not determined.

Methanocalculus, *M. pumilus*, was isolated from a landfill (Mori *et al*, 2000). Interestingly, no clones analysed from the two Brogborough samples, or the Mucking enrichment culture, grouped within the *Methanocorpusculaceae* cluster.

The *Methanomicrobiaceae* cluster contains *mcrA* sequences from *Methanoculleus bourgensis*, *Methanoculleus thermophilus*, *Methanofollis liminatans*, *Methanomicrobium mobile* and the unidentified *Methanomicrobiaceae* strain EBac. This cluster also contains sequences from all five landfills, including both Brogborough samples. Within the *Methanomicrobiaceae* cluster, sequences from the landfill samples were closely affiliated to *M. bourgensis* and *M. thermophilus*. No clones were identified that showed close affiliation to *M. liminatans* using any of the methods of tree construction. One clone, BSD28, showed affiliation to *M. mobile* on the Neighbor-Joining and Parsimony trees, but not on the Fitch distance-matrix tree.

The topology of the *Methanomicrobiaceae* cluster was slightly different in the Fitch distance-matrix and Neighbor-Joining trees. However, the topology of this cluster was significantly different in the Parsimony tree, compared to the other two trees. In particular, it was observed that four sequences, *M. thermophilus* and uncultured *Archaea* 80B, 90C and 91B, formed a cluster on the Parsimony tree, but not in the other two trees. These four sequences were all short by two to five amino acids at the C-terminal end. This phenomenon of clustering of sequences that were missing amino acids was also observed in the Parsimony trees of the *Methanobacteriales* and *Methanosarcinales* (Figures 4.6 and 4.12). In Figure 4.6, the sequences of uncultured archaeon 20B and the unidentified *Methanobacteriales* sp. grouped together, but they did not group together in Figures 4.4 or 4.5. In Figure 4.12, the sequences from

uncultured *Archaea* 26A and 94D, and the sequences of uncultured methanogens RS-ME42 and RS-ME43, formed a cluster. These sequences were not grouped together in either Figure 4.10 or 4.11. To determine if the observed phenomenon was due to the missing amino acids, these positions were filled in with consensus amino acids. When the phylogenetic analyses were performed with these filled in sequences, the clustering of these sequences on the Parsimony trees was no longer observed. The Fitch distance-matrix and Neighbor-Joining methods did not appear to be affected by the missing amino acids.

Three sequences from the Mucking enrichment culture grouped with *M. hungatei*. This cluster was distinct from the *Methanomicrobiaceae* cluster, which supports the separation of the genus *Methanospirillum* into different family, as proposed by Boone *et al* (1993). Interestingly, two clones, BSD63 and OS48, showed affiliation to the *Methanospirillaceae* cluster on some of the trees, but affiliation to different clusters on other trees.

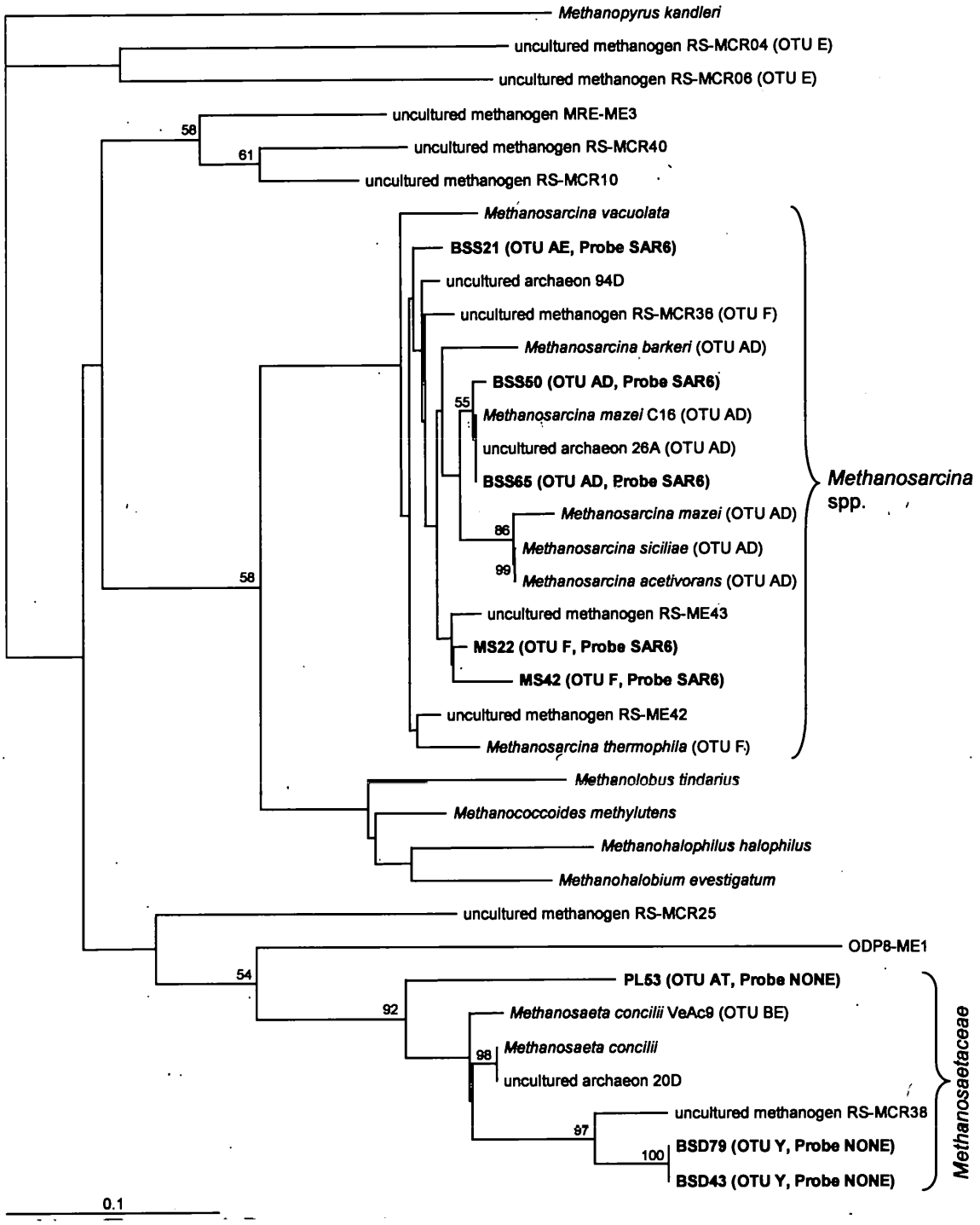
A large cluster of clones did not show affiliation to any *mcrA* sequences from described methanogen species. However, this cluster was clearly within the phylogenetic radiation of the *Methanomicrobiales*, and was named 'unidentified landfill *Methanomicrobiales mcrA*, cluster 1'. This cluster contained mostly clones from the Poyle leachate sample, but also clones from the Brogborough 3m sample and Hermitage leachate sample. The sequences in this cluster were distinct from the other *Methanomicrobiales* sequences because of their length, 139 amino acids against 146 amino acids.

Clones from the Poyle and Brogborough 3m samples formed two further clusters that did not show affiliation to any described species. Unidentified landfill *Methanomicrobiales mcrA* cluster 2, contained clones from the Poyle and Brogborough 3m samples, while cluster 3 contained only sequences from the Poyle sample. However, the *Taq*I-RFLP group, OTU AP, which was represented in cluster 3, was also detected in the Hermitage leachate sample. In all three trees, clusters 2 and 3 showed closer affiliation to each other than either did to any of the other clusters of *Methanomicrobiales*.

4.2.2.5 *Methanosarcinales* in landfill

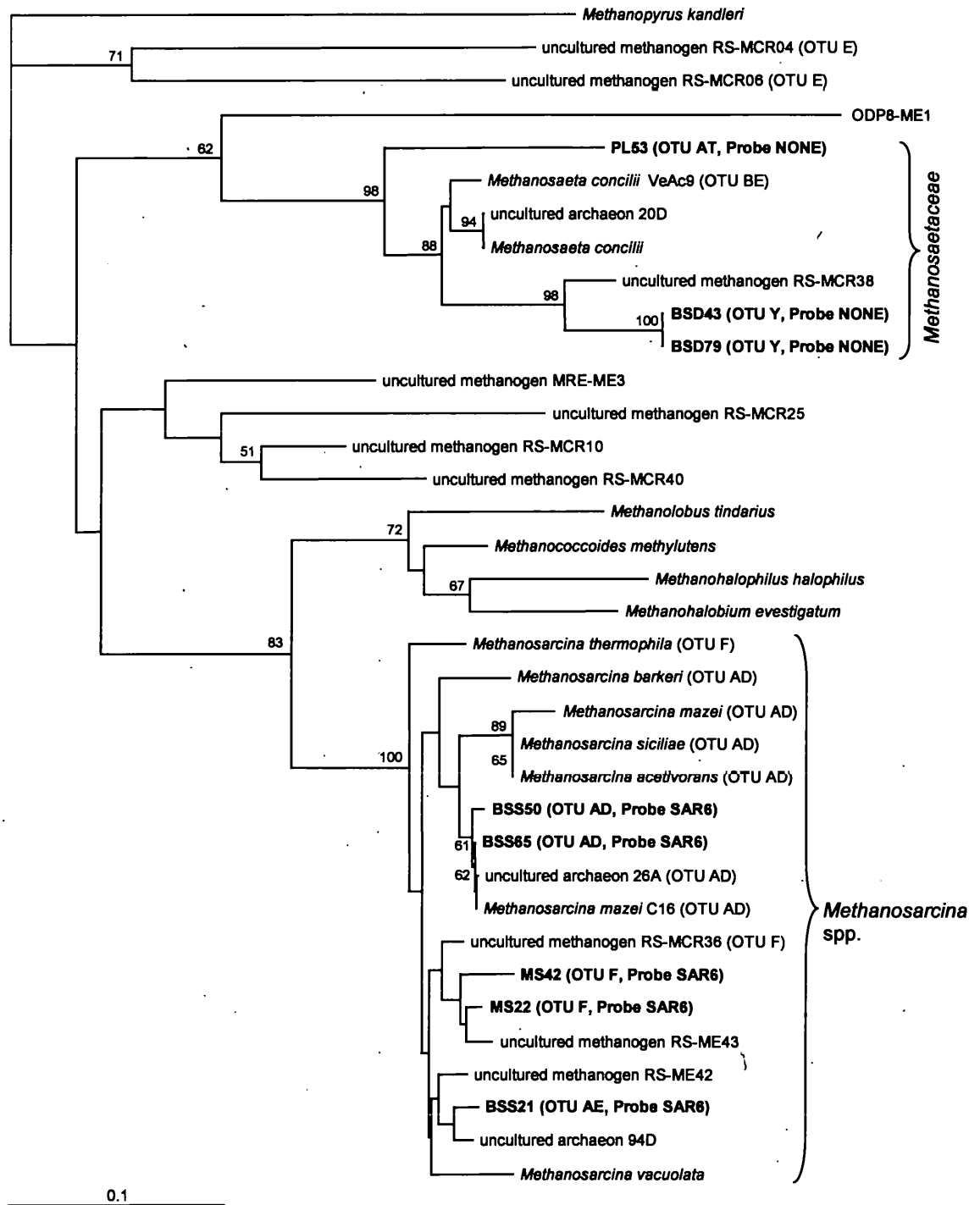
Nine clones were sequenced that grouped with the *Methanosarcinales*. The phylogenetic affiliations of eight of these clones are shown in Figures 4.10 to 4.12. Five of the eight clones grouped with species of the genus *Methanosarcina*. These five clones were from the Mucking and Brogborough 3m samples. The amino acid sequence of one clone, BSS65, was identical to that of *Methanosarcina mazei* strain C16. A second clone, BSS50 differed from *M. mazei* C16 by one amino acid. The other three landfill clones in the *Methanosarcina* cluster did not appear to be closely affiliated to any one of the seven described species of *Methanosarcina*. Two sequences from the Mucking enrichment culture, MS22 and MS42 were closely affiliated to a sequence from an uncultured methanogen, RS-ME43, from rice field soil. The two landfill clones had 97-99% amino acid sequence identity to the sequence from rice field soil. The clone BSS21 was closely affiliated to uncultured archaeon 94D from an anaerobic digester.

Figure 4.10 Fitch distance-matrix tree of cloned *mcrA* PCR products from landfill that show affiliation with species of the order *Methanosarcinales*.



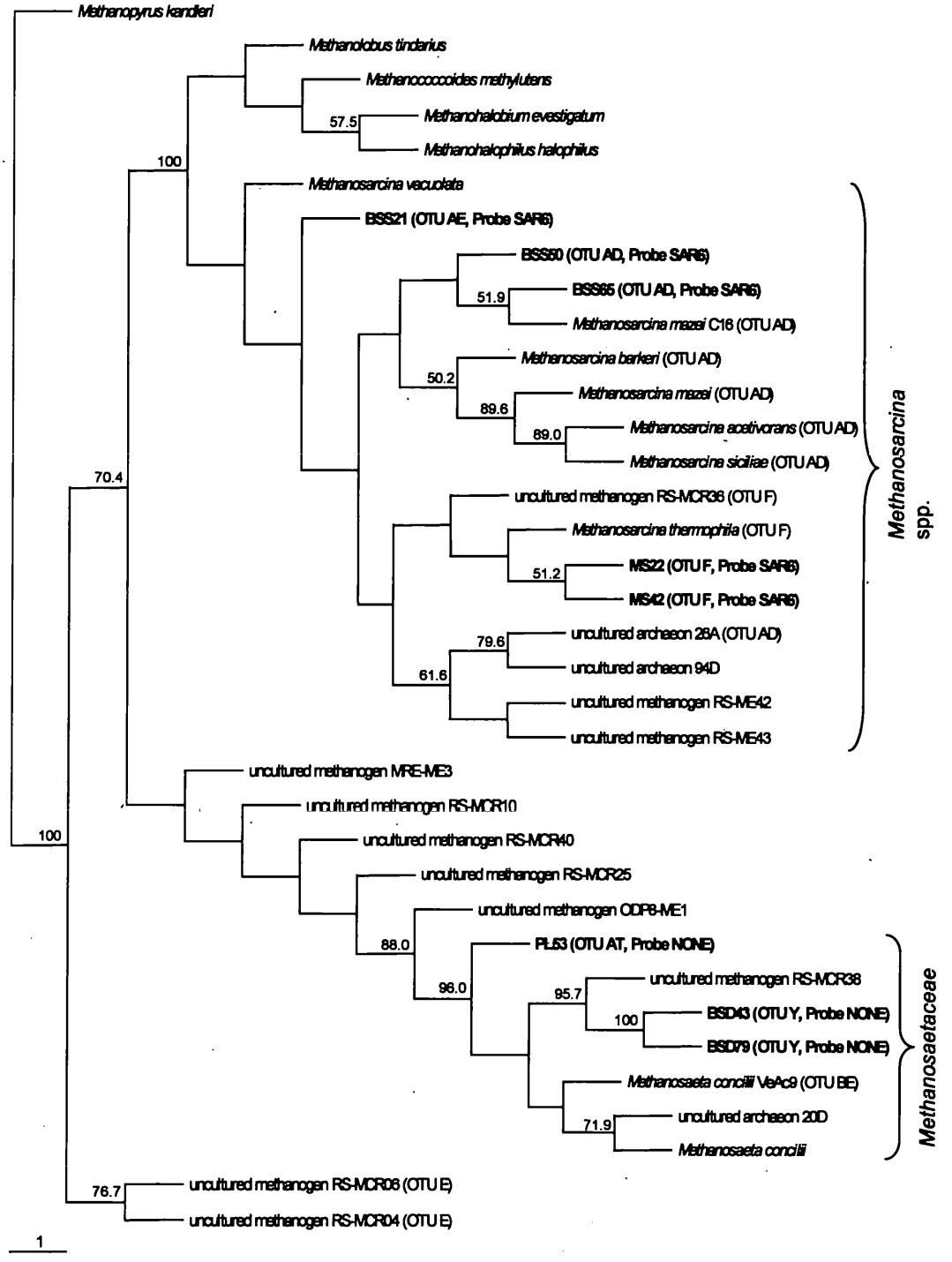
Legend: The phylogenetic tree was constructed from predicted *mcrA* amino acid sequences (146 positions) using Fitch distance-matrix analysis, as described in section 2.12. The sources of the *mcrA* sequences for described species are listed in Table 4.1. *Methanopyrus kandleri* is used as the outgroup. The bootstrap values above 50% are shown at nodes. BSS, BSD, MS and PL indicate cloned *mcrA* PCR products from the Brogborough 3m, Brogborough 18m, Mucking and Poyle landfill samples respectively (bold face). The OTUs as determined by *Taq*I RFLP (see Tables 3.3 and 4.1), and the *mcrA* probes hybridising to the landfill sequences, are indicated after each clone. NONE = not detected by any probe. N/D = not determined.

Figure 4.11 Neighbor-joining tree of cloned *mcrA* PCR products from landfill that show affiliation with species of the order *Methanosarcinales*.



Legend: The phylogenetic tree was constructed from predicted *mcrA* amino acid sequences (146 positions) using neighbor-joining analysis, as described in section 2.12. The sources of the *mcrA* sequences for described species are listed in Table 4.1. *Methanopyrus kandleri* is used as the outgroup. The bootstrap values above 50% are shown at nodes. BSS, BSD, MS and PL indicate cloned *mcrA* PCR products from the Brogborough 3m, Brogborough 18m, Mucking and Poyle landfill samples respectively (bold face). The OTUs as determined by *TaqI* RFLP (see Tables 3.3 and 4.1), and the *mcrA* probes hybridising to the landfill sequences, are indicated after each clone. NONE = not detected by any probe. N/D = not determined.

Figure 4.12 Parsimony tree of cloned *mcrA* PCR products from landfill that show affiliation with species of the order *Methanosarcinales*.



Legend: The phylogenetic tree was constructed from predicted *mcrA* amino acid sequences (146 positions) using parsimony analysis, as described in section 2.12. The sources of the *mcrA* sequences for described species are listed in Table 4.1. *Methanopyrus kandleri* is used as the outgroup. The bootstrap values above 50% are shown at nodes. BSS, BSD, MS and PL indicate cloned *mcrA* PCR products from the Brogborough 3m, Brogborough 18m, Mucking and Poyle landfill samples respectively (bold face). The OTUs as determined by *Taq*I RFLP (see Tables 3.3 and 4.1), and the *mcrA* probes hybridising to the landfill sequences, are indicated after each clone. NONE = not detected by any probe. N/D = not determined.

Three clones from landfill showed affiliation to two strains of *Methanosaeta concilii* (Figures 4.10 – 4.12). These clones were tentatively identified as *Methanosaetaceae*. Two identical clones from the Brogborough 18m sample, BSD43 and BSD79 were most closely related to an uncultured methanogen, RS-MCR38, from rice field soil, (94% amino acid sequence identity). The third landfill clone in the *Methanosaetaceae* cluster, PL53, from the Poyle leachate sample, had 86% amino acid sequence identity to the sequence from *M. concilii*. The topology of the *Methanosaetaceae* cluster was similar using each of the tree-construction methods, and it was supported by bootstrap values greater than 90% at several of the nodes.

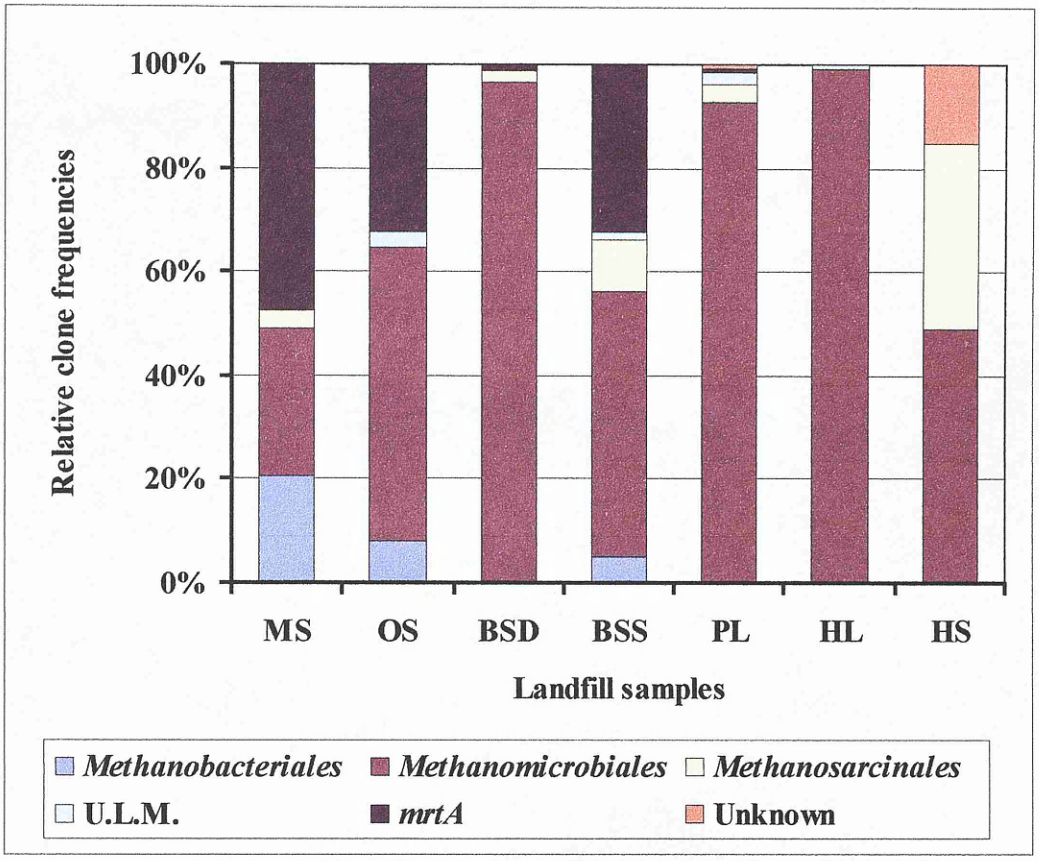
4.2.3 Comparison of the phylogenetic analyses with the results of the PCR-RFLP analyses

The 147-landfill *mcrA* clones that were sequenced were selected from each of the OTUs that were identified from the PCR-RFLP analysis (chapter 3). More than one clone was sequenced from each OTU, except those that contained only a single clone. Phylogenetic analysis of the clones was used to determine if the OTUs, defined by their *Taq*I restriction fragment pattern, corresponded to groups of closely related sequences based on their phylogenetic positioning. Figures 4.1 to 4.12 illustrate that the majority of OTUs were specific to groups of closely related clones and described species. For example, OTU AD was specific to a cluster of *Methanosarcina* spp and closely related clones from landfill (Figures 4.10 – 4.12). OTU B was specific to sequences in the cluster of unidentified landfill *Methanobacteriaceae mcrA* (Figures 4.4 – 4.6). Several clones were sequenced from the four most abundant OTUs (14 from OTU A, 23 from OTU D, 8 from OTU G and 4 from OTU U). Phylogenetic analysis of these clones indicated that OTU A contained clones that were distributed

through several clusters, all of which were affiliated with *Methanomicrobiales* (Figures 4.7 – 4.9). All the sequences from OTU D grouped within the cluster containing the *mrtA* sequence from *Methanobacterium formicicum* (Figures 4.4 – 4.6). However, the *Taq*I-RFLP that defined OTU D was common to *Methanococcus jannaschii mcrA* and *M. formicicum mrtA*. Most of the sequences from OTU G grouped within the cluster containing sequences of the genus *Methanocorpusculum* (Figures 4.7 – 4.9). However, OTU G also contained sequences that grouped with other clusters affiliated with members of the *Methanomicrobiales*. All four clones sequenced from OTU U grouped with the *Methanomicrobiaceae* cluster.

The results of the phylogenetic analyses were used to classify each of the OTUs into the following groups: *Methanobacteriales, Methanomicrobiales, Methanosarcinales, mrtA*, the unidentified landfill methanogens group and unknown group. The relative abundance of each of these groups was plotted for each clone library (Figure 4.13). This analysis showed that *Methanomicrobiales* were the dominant group in all the clone libraries, except for the Mucking enrichment culture. Figure 4.13 also shows that *mrtA* sequences were more abundant than *Methanobacteriales mcrA* sequences in all the clone libraries in which these two groups were detected. *Methanobacteriales mcrA* sequences were only detected in the Mucking, Odcombe and Brogborough 3m samples. *Methanosarcinales* were only detected in the Mucking, Brogborough 3m, Brogborough 18m, and Hermitage excavated refuse samples, and the Poyle leachate sample. The unidentified landfill methanogens group was detected in the Odcombe, Brogborough 3m, Poyle leachate and Hermitage leachate samples.

Figure 4.13 Relative abundance of methanogen groups in seven clone libraries.



Legend: Phylogenetic analysis of *mcrA*/*mrtA* sequences was used to classify the OTUs (defined by PCR-RFLP analysis) into six groups. The relative abundance of the six groups in the clone libraries for each landfill sample was plotted.
U.L.M. = unidentified landfill methanogens group.
Unknown = OTUs that could not be placed in any of the other five groups were classified as unknown. Some OTUs could not be classified because representative clones had not been sequenced.

Landfill samples:
MS – Mucking sample
OS – Odcombe sample
BSD – Brogborough 18m sample
BSS – Brogborough 3m sample
PL – Poyle sample
HL – Hermitage leachate sample
HS – Hermitage excavated refuse sample

4.3 DISCUSSION

Phylogenetic analysis of PCR-amplified gene sequences is probably the most common method of identifying uncultured microorganisms in the environment. By using phylogenetic methods to compare gene sequences from described species and organisms in the environment, it is possible to establish the phylogenetic type (phylotype) of uncultured organisms (Hugenholtz and Pace, 1996; Stahl, 1997). As stated earlier (section 4.1), the sequence information most commonly extracted from environmental samples is that for 16S rDNA. However, in this study a 'functional marker gene', *mcrA*, was used to specifically target the methanogenic *Archaea*.

The product of the *mcr* operon, methyl coenzyme-M reductase, appears to be unique to methanogens (Reeve *et al*, 1997b; Thauer, 1998). The use of *mcrA* as a phylogenetic tool for some methanogen orders has been demonstrated (Elberson and Sowers, 1997; Kudo *et al*, 1998; Lueders *et al*, 2001; Springer *et al*, 1995). Kudo *et al* (1998), Lueders *et al* (2001) and Springer *et al* (1995) compared methanogen phylogenies based on *mcrA* and 16S rRNA. They showed that the topology of the trees from both genes was similar. Comparison of the phylogenies based on *mcrA* and 16S rRNA, generated in this study, both supported the conclusions of these previous studies and extended them to encompass all methanogen orders so far described.

In this study, a 464 – 491bp fragment of the *mcrA* gene was amplified from seven samples from landfills. Clone libraries were generated from the PCR products and 632 clones were screened by PCR-RFLP analysis. Clones were selected from each of the operational taxonomic units (OTUs) identified by the PCR-RFLP analysis, and 147 clones were sequenced. Phylogenetic analysis of these sequences identified the

phylotypes of the methanogens in the landfill samples by their affiliation to *mcrA* sequences from described species. However, the published *mcrA* sequences from described species were not sufficient to identify all the phylotypes in landfill samples. In particular, the order *Methanomicrobiales* was represented by just three *mcrA* sequences, from *Methanomicrobium mobile*, *Methanofollis liminatans* and strain EBac, an unidentified *Methanomicrobiaceae*. To overcome this problem, the same *mcrA* gene fragment was amplified and sequenced from 12 species of methanogen. Recently, the *mcrA* sequences from *Methanoculleus thermophilus* and *Methanospirillum hungatei* have been determined (Lueders *et al*, 2001).

The addition of nine new *mcrA* sequences from described methanogen species, to the database of *mcrA* sequences, enabled the tentative identification of six phylotypes of methanogens in the landfill samples. The six phylotypes were related to *Methanobacterium formicicum mrtA*, *Methanobrevibacter arboriphilicus*, *Methanocorpusculum* spp, *Methanoculleus bourgensis*, *Methanospirillum hungatei* and *Methanosaeta concilii*. In addition, the new sequences from described species provided further evidence for the efficacy of the *mcrA* gene as a phylogenetic marker for the methanogenic *Archaea*.

Phylotypes related to published *mcrA* sequences from *Methanobacterium formicicum*, *Methanoculleus thermophilus* and *Methanosarcina* spp were identified in the landfill samples. In addition, the phylogenetic analyses revealed five clusters of *mcrA* sequences from the landfill samples, which did not appear affiliated to any of the described species. Three of these clusters grouped within the phylogenetic radiation of the *Methanomicrobiales*. One cluster grouped within the phylogenetic radiation of

the *Methanobacteriales*. The fifth cluster did not appear to group within the phylogenetic radiation of any of the five orders of methanogenic *Archaea*.

4.3.1 *Methanomicrobiales* in landfill

Methanomicrobiales sequences were dominant in six of the seven clone libraries, both in terms of numbers of clones and genetic diversity. In the clone libraries of the Poyle and Hermitage landfill leachate samples and the Brogborough sample excavated from 18m depth, *Methanomicrobiales* sequences accounted for greater than 90% of the clones. The Mucking clone library was the only one in which *Methanomicrobiales* were not the dominant group. However, this clone library was generated from an enrichment culture grown in a *Methanobacterium* medium, which would be expected to select for *Methanobacterium* spp.

Methanomicrobiales have been detected as the dominant methanogens in a mesophilic biogas reactor treating manure and industrial organic waste (Hansen *et al*, 1999), and a wood-fermenting anaerobic bioreactor (Macario *et al*, 1991). However, neither of these studies indicated the diversity within the *Methanomicrobiales*. Previous studies have detected *Methanomicrobiales* in landfill (Fielding and Archer, 1986; Fielding *et al*, 1988; Luton, 1996; Mori *et al*, 2000). Luton (1996) used an oligonucleotide probe that hybridised to the *mcrA* gene from *M. bourgensis, M. hungatei* and *M. liminatans*, to detect these species in landfill leachate. Fielding *et al* (1988) isolated a coccoid methanogen from landfill that was unrelated to any of the reference methanogens used to raise antibody probes. It was concluded that this unidentified methanogen was probably a *Methanogenium* sp or *Methanocorpusculum* sp. Mori *et al* (2000) isolated

and characterised a new species from landfill, *Methanocalculus pumilus*, belonging to the family *Methanocorpusculaceae*.

A number of studies have identified *Methanomicrobiales* by phylogenetic analysis of PCR amplified 16S rDNA sequences. Whitehead & Cotta (1999) identified sequences related to *Methanoculleus marisnigri* and *M. parvum* in swine waste storage pits. Munson *et al* (1997) identified sequences in samples from coastal salt marshes related to *Methanoculleus* spp and *Methanogenium* spp. Lueders & Friedrich (2000) identified sequences related to *Methanoculleus thermophilus* in rice field soil. Hales *et al* (1996) identified sequences in blanket peat bog that showed closest affiliation to *M. hungatei*. Lueders *et al* (2001) identified methanogens in rice field soil by phylogenetic analysis of PCR-amplified *mcrA* sequences. However, they failed to detect any sequences related to *Methanomicrobiales*. None of these studies has identified the level of diversity of *Methanomicrobiales*, which has been detected in this study.

The order *Methanomicrobiales* contains four families of hydrogenotrophic methanogens (Table 1.5). Most species are able to utilise formate as a substrate for methanogenesis, and certain species can use alcohols to reduce CO_2 to methane (Boone *et al*, 1993). The majority of *Methanomicrobiales* are mesophilic and grow at neutral pH (Table 1.5). The cell walls are proteinaceous and the cells of certain species are lysed easily by detergents (Garcia *et al*, 2000). Differences in the ease of lysing the cells of different species could lead to bias in the representation of different groups in the clone libraries. A physical lysis method was used in this study because it is less likely to suffer from this limitation.

Unidentified *Methanomicrobiales* in landfill

Three clusters of sequences were detected from the landfill samples that grouped within the phylogenetic radiation of the *Methanomicrobiales*, but did not show close affiliation to any of the described species. Using Fitch distance-matrix and Neighbor-Joining analyses all three clusters were deeper-branching than the clusters containing described species of *Methanomicrobiales*. Several genera from the *Methanomicrobiales* were not represented in the phylogenetic analyses. These were *Methanolacina, Methanogenium, Methanocalculus* and *Methanoplanus*. *Methanoplanus* is the only genus in the family *Methanoplanaceae* (Ollivier *et al*, 1997). Phylogenetic analysis based on 16S rDNA, as shown in Figure 1.8, do not show any of the four genera as deeper-branching than members of the *Methanospirillaceae, Methanocorpusculaceae* or *Methanomicrobiaceae*. This suggests that the unidentified *Methanomicrobiales* clusters may represent novel genera or families within the *Methanomicrobiales*.

4.3.2 *Methanobacteriales* in landfill

The order *Methanobacteriales* is an order of mainly rod-shaped methanogens that grow by CO_2 reduction. *Methanobacteriales mcrA* sequences were detected only in the Mucking, Odcombe and Brogborough 3m clone libraries. However, the isogene of *mcrA, mrtA*, was detected in these three clone libraries, and in the Brogborough 18m and Poyle libraries. *MrtA* has only been detected in *Methanothermus fervidus, Methanothermobacter thermoautotrophicus, Methanobacterium bryantii, Methanobacterium formicicum* and *Methanococcus jannaschii* (Bult *et al*, 1996; Lehmacher and Klenk, 1994; Luton, 1996; Smith *et al*, 1997). Phylogenetic analysis

of sequences amplified from landfill revealed sequences related to *mrtA* from *M. formicicum* and uncultured *Archaea* from anaerobic digesters. None of the landfill sequences were affiliated to *mrtA* from *M. jannaschii*. It was assumed therefore, that all the *mrtA* sequences from landfill were from *Methanobacteriales*. However, *mrtA* sequences were detected more frequently than *mcrA* sequences from *Methanobacteriales*, in all the landfill clone libraries in which either phylotype was detected. Some possible explanations for this discrepancy are PCR bias in favour of *mrtA* sequences, presence of *mrtA* sequences from non-*Methanobacteriales* and/or mis-classification of clones. Ninety-five percent of *mrtA* clones belonged to OTU D. The *Taq*I-RFP for OTU D consisted of a single fragment of 464bp; i.e. there were no *Taq*I restriction sites in the PCR products of OTU D. Twenty-three out of 86 clones from OTU D were sequenced and all the sequences were affiliated to *M. formicicum mrtA*. However, it is possible that some of the clones that were not sequenced were not related to *M. formicicum*.

Reeve *et al* (1997b) concluded that the presence of two methyl reductase enzymes in methanogens could lead to either of the enzymes becoming non-essential for methanogenesis. A non-essential enzyme is likely to evolve at a different rate from an essential enzyme. The implication of this is that different phylogenies might be produced from the two enzymes. All *mrtA* sequences are most similar to *mcrA* sequences from *Methanococcales*. It is believed that the *Methanobacteriales mrt* genes were obtained by lateral transfer from the *Methanococcales* (Reeve *et al*, 1997b). *MrtA* may be present in the other methanogen orders, but not amplifiable with the PCR primers used in this study.

Methanobacterium spp in landfill

Based on the phylogenetic analyses, one cluster of landfill *mcrA* sequences was tentatively identified as *Methanobacterium* spp. This cluster contained sequences from three of the landfill clone libraries. The described species to which this cluster showed closest affiliation was *Methanobacterium formicicum*. Clones with identical amino acid sequences to *M. formicicum mrtA* were also identified. *Methanobacterium* spp, and in particular *M. formicicum*, have been detected previously in landfill. *M. formicicum* was isolated from landfill samples as an endosymbiont of the ciliate protozoan *Metopus palaeformis* (Finlay and Fenchel, 1991). It was also detected in landfill leachate using oligonucleotide probes for the *mcrA* gene (Luton, 1996). Fielding *et al* (1988) identified *Methanobacterium* spp isolated from landfill using immunological techniques.

The genus *Methanobacterium* contains 13 species (Table 1.5), all of which are able to grow on H_2+CO_2. In addition, six species, including *M. formicicum*, can use formate, and three species, including *M. formicicum*, are able to use certain alcohols. Most members of the genus are mesophilic, but four species are thermophilic with optimum growth temperatures in the range 55-65°C (Table 1.5). The broad range of G+C values determined for the genus *Methanobacterium*, 29 to 62 mol%, indicate that the genus is composed of more than one genus (Garcia *et al*, 2000). This conclusion is further supported by the observation that the *mcrA* sequences of *Methanobacterium bryantii* and *M. formicicum* do not form a monophyletic cluster using any of the methods of tree-construction (Figures 4.4 – 4.6). The position of *M. bryantii* in relation to *M. formicicum*, the *Methanobrevibacter* spp and the *Methanothermobacter* spp is different in each tree. Sequencing of the *mcrA* gene from the remaining 11

species of *Methanobacterium* might provide a more precise identification of the cluster of landfill *mcrA* sequences.

Methanobrevibacter spp in landfill

A single sequence closely related to *Methanobrevibacter arboriphilicus* was identified from the Odcombe landfill. Members of the genus *Methanobrevibacter* grow at mesophilic temperatures and neutral pH, although the optimum pH for *M. arboriphilicus* is 7.8-8.0 (Garcia, 1990; Garcia *et al*, 2000). All members of the genus are able to use H_2+CO_2 as substrates for methanogenesis, and some species can utilise formate (Sowers, 1995). *Methanobrevibacter* spp have been isolated from a wide variety of environments, though each species inhabits a specialised habitat (Garcia *et al*, 2000). *M. arboriphilicus* was isolated from the wetwood of living trees (Zeikus, 1977). Microorganisms antigenically related to *M. arboriphilicus* have been detected in an anaerobic sludge blanket reactor treating wastewater from a sugar plant (Grotenhuis *et al*, 1991). A 16S rDNA sequence related to *Methanobrevibacter smithii* was detected in swine waste storage pits (Whitehead and Cotta, 1999). (Luton, 1996) failed to detect *M. ruminantium* in landfill leachate using oligonucleotide probes for the *mcrA* gene.

Unidentified *Methanobacteriales* in landfill

A cluster of highly similar sequences from three landfills was detected that grouped within the phylogenetic radiation of the *Methanobacteriaceae*, but was not closely affiliated to any described species. There are 17 described species within the family *Methanobacteriaceae,* for which the sequence of *mcrA* has not been determined. These species include: 11 *Methanobacterium* spp, five *Methanobrevibacter* spp and

one *Methanosphaera* sp. The unidentified landfill *Methanobacteriaceae mcrA* sequences could be related to one of these species, or they could represent a novel genus. The most closely related sequences to these unidentified landfill sequences were from uncultured *Archaea* detected in anaerobic digesters (Hougaard and Westermann, 2000). A sequence has been determined for *mcrA* from *Methanosphaera stadtmanae* (Luton, 1996). However, phylogenetic analyses (Figures 4.1 – 4.6) indicate that this sequence is more closely related to *mrtA* than *mcrA*.

4.3.3 *Methanosarcinales* in landfill

The order *Methanosarcinales* groups all the acetotrophic and/or methylotrophic methanogens into two families (Garcia *et al*, 2000). Sequences affiliated to species of the genus *Methanosarcina* and the family *Methanosaetaceae* were detected in landfill samples. *Methanosarcina* and *Methanosaeta* are the only genera of methanogens able to utilise acetate as a substrate for methanogenesis. (Ferry, 1992) stated that about two-thirds of the methane produced in nature originates from the methyl group of acetate. Acetate concentrations in landfill are typically in the mM range, well above the threshold concentration for acetate utilisation by *Methanosarcina* spp and *Methanosaeta* spp (Anon, 1988; Grosskopf *et al*, 1998; Maule *et al*, 1994). The threshold concentration is that below which an organism is no longer able to degrade a particular substrate. However, the results of this study appear to indicate that hydrogenotrophic methanogens are dominant in landfills.

Methanosarcina spp in landfill

The genus *Methanosarcina* includes organisms that grow on methylotrophic substrates, such as methanol, methylamines or methyl sulphides, and sometimes acetate or H_2 + CO_2 (Table 1.5). The seven described species of *Methanosarcina* were isolated from fresh water and marine sediments, as well as anaerobic digesters. The genus *Methanosarcina* predominates in many anaerobic ecosystems where organic matter is completely degraded to CH_4 and CO_2 (Garcia *et al*, 2000). *Methanosarcina* spp were detected in four of the landfill samples. They accounted for 36% of clones in the clone library from the Hermitage excavated refuse sample. In the other clone libraries, *Methanosarcina* spp accounted for 0 – 10% of the clones. There are only two previous reports of *Methanosarcinales* in landfill. Fielding *et al* (1988) detected *Methanosarcina barkeri* in landfill using antibody probes, and Luton (1996) detected *M. barkeri* in landfill leachate using oligonucleotide probes hybridising to *mcrA*. The *Methanosarcina*-like clones from the landfill samples consist of sequences most closely related to *M. mazei* strain C16 and to uncultured methanogens from rice field soil and anaerobic digesters (Figures 4.10 - 4.12).

Methanosaetaceae in landfill

The family, *Methanosaetaceae* includes all the obligate acetotrophic methanogens grouped into the genus *Methanosaeta*, which currently consists of two species (Garcia *et al*, 2000). *Methanosaeta* spp have been shown to be one of the dominant methanogenic groups in anoxic rice field soil and anaerobic digesters (Fey and Conrad, 2000; Grosskopf *et al*, 1998; Raskin *et al*, 1995; Sekiguchi *et al*, 1999). However, only four clones were identified from two landfill samples that grouped with *Methanosaeta concilii*.

4.3.4 Novel methanogenic lineage in landfill

A deep-branching cluster of *mcrA* sequences was detected in landfill that was related peripherally to the orders *Methanosarcinales* and *Methanomicrobiales*. These sequences were from two landfills, Odcombe and Hermitage. Clones with the same *Taq*I-RFP were also detected in the Brogborough and Poyle landfills. A *mcrA* sequence was identified in GenBank that grouped with this cluster of landfill clones. This sequence was from an uncultured archaeon from an anaerobic digester (Hougaard and Westermann, 2000). However, the only information available about this sequence is that contained in the GenBank entry. Hinrichs *et al* (1999) detected a cluster of 16S rRNA sequences in marine sediments that occupied a similar position in the methanogen phylogeny. This cluster accounted for 148 of the 176 archaeal clones recovered from the sediment samples. It is possible that the 16S rRNA sequences from marine sediment and the *mcrA* sequences from landfill are from the same novel group of uncultured methanogens. One way to confirm this would be to isolate the organisms in pure culture and sequence both the 16S rRNA and *mcrA* genes. However, the lack of close relatives to these sequences among the described species of methanogens, makes difficult the prediction of suitable culture conditions.

4.3.5 Detection of chimeric sequences

The amplification of chimeric sequences in multi-template PCR can lead to the overestimation of community diversity or to the description of non-existent species (Kopczynski *et al*, 1994). The detection of chimeric sequences has been reported in a number of studies (Godon *et al*, 1997a; Godon *et al*, 1997b; Munson *et al*, 1997). Lueders *et al* (2001) reported the detection of four chimeric or truncated sequences

among PCR amplified *mcrA* sequences from rice field soil. However, they did not give any details of how the chimeric sequences were detected. One method that has been used successfully to detect chimeric sequences, involves comparing the phylogenetic affiliations of sequences based on regions from the 5' and 3' ends (Godon *et al*, 1997a; Kopczynski *et al*, 1994; Munson *et al*, 1997). Sequences whose phylogenetic positions change significantly between the trees may be chimeric.

Application of this method to cloned *mcrA/mrtA* sequences from landfill samples, as described in section 2.12, identified several potentially chimeric clones. For example, clone OS48 grouped with the *Methanospirillaceae* cluster in the trees constructed with the whole sequence (440 bases) and the 5' 200 bases (data not shown). Whereas, in the tree constructed with the 3' 200 bases, clone OS48 grouped with the *Methanocorpusculaceae*. Several other clones showed less radical position changes; i.e. they remained in the same cluster, but changed position within that cluster.

The detection of chimeric sequences can be difficult, particularly when the chimeras are formed from highly similar sequences. Chimera detection is always likely to be a problem with PCR-based investigations of microbial diversity. One solution would be to use PCR-based and non-PCR-based methods in conjunction.

4.3.6 Isolation of landfill methanogens

Isolation of microorganisms in pure culture and subsequent characterisation is the typical procedure for confirming the discovery of a new species, or the presence of a previously described species in the ecosystem under investigation (Elberson and Sowers, 1997; Kim *et al*, 1996; Lomans *et al*, 1999; Ni and Boone, 1991). An

attempt was made to isolate landfill methanogens in pure culture, by inoculating a series of anaerobic cultures with dilutions of leachate from a model landfill reactor, as described in section 2.15. One culture was identified by PCR-RFLP analysis that appeared to contain a single OTU.

A 750bp fragment of the 16S rDNA was amplified from the culture by PCR using the *Archaea* specific primer pair 0348a Forward and 1100a Reverse (Table 2.2). Examination of the results of automated sequencing of the PCR product appeared to indicate that the 16S rDNA from more than one species had been amplified. However, a 206bp section of the sequence was unambiguous, and this was used to interrogate the GenBank database using BLAST. The sequence of this fragment was identical to three 16S rDNA sequences from methanogens. These sequences were *Methanobacterium formicicum* strain DSM 1312 16S rRNA gene (GenBank accession no. M36508), *Methanobacterium* sp strain BRM12 16S rRNA gene (GenBank accession no. X99137) and *Methanomicrobium* sp strain BRM9 16S rRNA gene (GenBank accession no. X99138). Although this last sequence is named *Methanomicrobium* sp, it is phylogenetically related to the genus *Methanobacterium*.

4.3.7 Summary

Phylogenetic analysis of partial amino acid sequences of methyl CoM reductase from described species and landfill clones showed the following:

- Methanogen phylogeny based on partial *mcrA* amino acid sequences showed good agreement with the phylogeny based on 16S rRNA.

- Sequencing and phylogenetic analysis of clones from the same OTU, determined by PCR-RFLP analysis, showed that the majority of OTUs were specific to one cluster of sequences.
- The phylogenetic analysis enabled the cloned *mcrA* sequences to be classified into 12 groups, seven of which were tentatively identified by their affiliation to described species.
- Phylogenetic analyses showed that *mrtA* was amplified in addition to *mcrA*.
- Sequences were identified that were affiliated to the orders *Methanobacteriales, Methanomicrobiales* and *Methanosarcinales*, but not *Methanococcales* or *Methanopyrales*. This agrees with previous investigations, which have identified members of the same methanogen orders in landfill (Fielding *et al*, 1988; Luton, 1996; Mori *et al*, 2000).
  - *Methanomicrobiales* was the most diverse and abundant group in landfill. *Methanomicrobiales* accounted for 29% - 99% of clones in the seven clone libraries. Sequences affiliated to the families *Methanocorpusculaceae, Methanomicrobiaceae* and *Methanospirillaceae* were identified. In addition, three clusters of sequences were identified that grouped within the phylogenetic radiation of the *Methanomicrobiales*, but were not affiliated to any of the described species used in the analysis.
  - *McrA* sequences were identified that appeared affiliated to *Methanobrevibacter arboriphilicus* and *Methanobacterium formicicum*. *MrtA* sequences were also identified that were closely related to *M. formicicum mrtA*. Clusters of *mcrA* and *mrtA* sequences were identified that were not closely affiliated to any of the described species used in the analysis. These

clusters showed closest affiliation to uncultured *Archaea* from anaerobic digesters.

- Sequences were amplified from the landfill samples that grouped with sequences from *Methanosarcina* spp and *Methanosaeta concilii*.
- A cluster of sequences was also identified that appeared related to, but distinct from the orders *Methanosarcinales* and *Methanomicrobiales*.

- Each landfill sample appears to contain a unique and diverse methanogen community, even samples taken from the same landfill.

5 Development and application of *mcrA* probes for the characterisation of methanogen communities in landfill

5.1 INTRODUCTION

Oligonucleotide probes are single strands of labelled DNA or RNA, which hybridise with a complementary nucleic acid sequence (target sequence) allowing detection of this sequence. Thus, oligonucleotide probes can be used to evaluate the presence of specific nucleic acid sequences (and the populations they represent) in samples obtained from environments, such as landfills. The use of such probes can provide a complete microbial community description without the need to cultivate the community members (Raskin *et al*, 1995). Probes can be designed that are specific to individual species or phylogenetically coherent groups of organisms, by targeting variable or conserved regions of a gene sequence. By this approach, it is generally possible to select the level of probe specificity (e.g., species or genus specific) (Raskin *et al*, 1994b).

Oligonucleotide probes have been designed that hybridise to the 16S rRNA of different groups of methanogens (Raskin *et al*, 1994b). These probes have been used to describe the methanogen communities in the gastrointestinal tracts of domestic animals, anaerobic digesters and landfill (Lin *et al*, 1997; Raskin *et al*, 1994a; Sekiguchi *et al*, 1999; M. van Dyke, personal communication). Probes have also been designed that target the *mcrA* gene of methanogens, and applied to the characterisation of methanogens in blanket bog peat and landfill (Hales *et al*, 1996;

Luton, 1996; McDonald *et al*, 1999). However, all of these probes were based on sequence information from microorganisms previously isolated in pure culture. Thus, they may not be free from cultivation limitation (Godon *et al*, 1997b). Also, the specificity of these probes was not optimal for an accurate description of the methanogen community in landfill, as revealed in this study by phylogenetic analysis of the *mcrA* gene (chapter 4).

This chapter describes the design and evaluation of group-specific oligonucleotide probes to characterise the methanogen community in landfill. The design of the group-specific probes was based on the results of the phylogenetic analysis of *mcrA* gene sequences from landfill samples (chapter 4). The evaluation of the probes by screening of *mcrA* clone libraries generated from landfill samples is described.

5.2 RESULTS

5.2.1 Design of group-specific oligonucleotide probes for the *mcrA* gene

The design of the oligonucleotide probes was based on sequences of *mcrA* PCR products cloned from landfill samples. Probes *mcrA*-BAC1, *mcrA*-BAC2, *mcrA*-MIC3, *mcrA*-MIC4, *mcrA*-MIC5, *mcrA*-SAR6, *mcrA*-ULM7 and *mcrA*-MIC8 were based on sequence data obtained from the Mucking and Odcombe landfills. Designs of the probes *mcrA*-BAC9, *mcrA*-MIC10, *mcrA*-MIC11/12 and *mcrA*-SAE13 were based on sequence data from the Mucking, Odcombe, Brogborough and Poyle landfills. Phylogenetic relationships of the cloned *mcrA* sequences, as described in chapter 4, were used to define target groups for the oligonucleotide probes (Table 5.1). Predicted amino acid sequences of *mcrA* were aligned and scanned by eye for

conserved amino acid regions unique to each target group. Oligonucleotides complementary to the corresponding nucleotide regions were synthesised and labelled with digoxigenin, as described in section 2.13.1. The sequences of 13 methanogen group-specific probes are given in Table 5.2.

Table 5.1 Target groups for *mcrA* probes

Target groups	Probes
Gene: *mcrA*	
ORDER I: *METHANOBACTERIALES*	
Family I: *Methanobacteriaceae*	
Genus I: *Methanobacterium*	} *mcrA*-BAC9
Genus II: *Methanobrevibacter*	} *mcrA*-BAC9
Unidentified landfill *Methanobacteriaceae*	} *mcrA*-BAC2 } *mcrA*-BAC9
ORDER II: *METHANOMICROBIALES*	
Family I: *Methanocorpusculaceae*	} *mcrA*-MIC3
Clones OS15, OS20, OS59, OS80	} *mcrA*-MIC4 } *mcrA*-MIC3
Family II: *Methanomicrobiaceae*	} *mcrA*-MIC5 } *mcrA*-MIC10
Clone OS77	} *mcrA*-MIC8 } *mcrA*-MIC5 } *mcrA*-MIC10
Family III: *Methanospirillaceae*	} *mcrA*-MIC5 } *mcrA*-MIC10
Unidentified landfill *Methanomicrobiales* cluster 2	} *mcrA*-MIC5 } *mcrA*-MIC10
Unidentified landfill *Methanomicrobiales* cluster 3	} *mcrA*-MIC5 } *mcrA*-MIC10
Unidentified landfill *Methanomicrobiales* cluster 1	} *mcrA*-MIC11/12
ORDER III: *METHANOSARCINALES*	
Family I: *Methanosarcinaceae*	
Genus I: *Methanosarcina*	} *mcrA*-SAR6
Family II: *Methanosaetaceae*	} *mcrA*-SAE13[a]
Unidentified landfill methanogens	} *mcrA*-ULM7
Gene: *mrtA*	
ORDER I: *METHANOBACTERIALES*	
Family I: *Methanobacteriaceae*	
Genus I: *Methanobacterium*	} *mrtA*-BAC1

Key: Target groups for oligonucleotide probes were defined from the phylogenetic relationships of cloned *mcrA* sequences from landfill (chapter 4). Hybridisation temperatures and stringency wash conditions were determined experimentally. The specificity of the probes for their target groups was evaluated by comparing the results from sequencing and phylogenetic analysis of *mcrA* PCR products with the results from probing dot blots of the same *mcrA* PCR products. The *mcrA*-reverse PCR primer was used as a universal probe for all *mcrA/mrtA* sequences.
[a] Probe *mcrA*-SAE13 has not been tested experimentally.

The probes were designed to encompass the diversity of *mcrA* sequences detected in landfill, as described in chapter 4. Two probes were designed to cover *mcrA* sequences from landfill grouping within the phylogenetic radiation of the *Methanobacteriales*. Probe *mcrA*-BAC2 was designed to detect sequences in the

unidentified landfill *Methanobacteriaceae* cluster, shown in Figures 4.4 – 4.6. Probe *mcrA*-BAC9 was designed to cover the sequences detected by probe *mcrA*-BAC2, plus sequences in the *Methanobacterium* and *Methanobrevibacter* clusters (Figures 4.4 – 4.6).

Table 5.2 Oligonucleotide probes for the *mcrA* gene.

Probe	Sequence 5' - 3'	Hybridisation temperature (°C)[a]
mcrA-BAC9	GGAATVACTGAAGCACCA	52
mcrA-BAC2	GGTGGACTCACGGAAGCACCA	68
mcrA-MIC10	ATGGACTACMTCMASGACAA	56
mcrA-MIC3	ATGGACTACCTCCATGACAAG	62
mcrA-MIC4	AGCCCGGCAAACAATGTTGCA	64
mcrA-MIC5	AAYGCMATGGARCAGTAC	50
mcrA-MIC8	GGCGGCTACTCGCAGGCACCA	72
mcrA-MIC11	CACGGCGCTCTCGGCAAG	62
mcrA-MIC12	CAYGGYGGYWTCGGCAAG	56
mcrA-SAR6	AAGTACAACGGTGCTGCAA	56
mcrA-SAE13	TTCGGCGGATTYGCMAAGGCA	64[b]
mcrA-ULM7	GGCTTCTGCAAGCTTGACCCG	68
mrtA-BAC1	GGTATCTGTGGAACCAAAGCA	62
mcrA-P3 reverse[c]	TTCATTGCRTAGTTWGGRTAGTT	60

Key: A = adenosine; C = cytidine; G = guanine; T = thymidine; M = A or C; R = A or G; S = C or G; W = A or T; Y = C or T; V = A, C or G.
[a] Hybridisation temperatures were determined experimentally.
[b] Probe *mcrA*-SAE13 has not been tested experimentally.
[c] The *mcrA*-P3 reverse PCR primer was used as a universal probe for all *mcrA/mrtA*.

Several probes were designed to encompass the groups identified within the order *Methanomicrobiales*. Probe *mcrA*-MIC3 is specific to the Methanocorpusculaceae cluster (Figures 4.7 – 4.9). Probe *mcrA*-MIC4 is specific to a small cluster of sequences isolated from landfill, within the cluster detected by probe *mcrA*-MIC3. Probe *mcrA*-MIC5 targets the *Methanomicrobiaceae* and *Methanospirillaceae* clusters. Probe *mcrA*-MIC8 was specific to single clone, OS77, detected by probe *mcrA*-MIC5. This clone was phylogenetically most closely affiliated to the *Methanomicrobiaceae*. However, it was distinct from the other

Methanomicrobiaceae because the amplified product was 470bp, compared to 491bp for the other *Methanomicrobiaceae*. In addition, it contained a unique region of amino acid sequence. Probe *mcrA*-MIC10 is a degenerate version of probe *mcrA*-MIC3, and was designed to encompass the groups detected by probes *mcrA*-MIC3, *mcrA*-MIC4 and *mcrA*-MIC5, plus the 'unidentified landfill *Methanomicrobiales* clusters 2 and 3' (Figures 4.7 – 4.9). Probe *mcrA*-MIC11 was designed using sequences from the Brogborough 3m sample, which formed part of 'unidentified landfill *Methanomicrobiales* cluster 1'. However, this probe contained up to four mismatches with sequences from the Poyle landfill that also grouped in the 'unidentified landfill *Methanomicrobiales* cluster 1'. Probe *mcrA*-MIC12 is a degenerate version of probe *mcrA*-MIC11, designed to encompass all the sequences in 'unidentified landfill *Methanomicrobiales* cluster 1'. Probes *mcrA*-MIC11 and *mcrA*-MIC12 were mixed together in a 1:16 molar ratio and used as a single probe. The hybridisation temperature used for probe *mcrA*-MIC11/12 was 56°C.

Two probes were designed to target landfill clones affiliated with species of the order *Methanosarcinales*. Probe *mcrA*-SAR6 was designed to hybridise to landfill clones affiliated with the genus *Methanosarcina*, as shown in Figures 4.10 – 4.12. Probe *mcrA*-SAE13 was designed to be specific to the *Methanosaetaceae* cluster.

The target group for probe *mcrA*-ULM7 was the cluster of 'unidentified landfill methanogens' shown in Figures 4.1 – 4.3.

MrtA, the isogene of *mcrA*, accounted for 14% of the clones in the seven clone libraries generated from landfill samples. Based on the results of the PCR-RFLP and

phylogenetic analyses (chapters 3 and 4), the majority of these clones showed closest affiliation to the *mrtA* sequence from *Methanobacterium formicicum*. Probe *mrtA*-BAC1 was designed to cover the *Metahnobacterium mrtA* cluster, shown in Figures 4.4 – 4.6.

5.2.2 Optimisation of hybridisation and wash conditions

Dot blots of *mcrA/mrtA* PCR products from landfill clone libraries were probed initially with 3pmol ml^{-1} of digoxigenin-labelled oligonucleotide, and with a hybridisation temperature 10°C below the melting temperature of the probe (Tm -10°C). The melting temperature was calculated using the formula: Tm ≐ 4(G+C) + 2(A+T). After hybridisation of the probe, the membrane was washed twice in 1× wash solution and twice in 0.5× wash solution, as described in section 2.13.3. These conditions resulted in false positive signals and a very dark background. Reducing the probe concentration to 1pmol ml^{-1}, increasing the hybridisation temperature to Tm -0°C and adding an extra washing step (two washes in 0.1× wash solution), greatly reduced the false positive signals and the dark background. The dark background was further reduced by the use of cling film in place of acetate sheets to wrap the membrane at the end of the chemiluminescent detection procedure (section 2.13.4).

5.2.3 Evaluation of specificity of probes for target groups

The specificity of the probes for their target groups was evaluated by comparing the results obtained from probing of 114 *mcrA* clones, with the phylogenetic position of the clones. The specificity of the probes for their target groups is illustrated in Figures 4.1 – 4.12 (chapter 4). The phylogenetic positions of 78 clones are displayed together with the probes that hybridised to those clones. Probing of the 114 reference

clones with 11 probes produced just 11 false positive results, indicating that overall; the probes were specific for their target groups.

The complementarity of the probes with the 114 reference clones was analysed. This showed that, under the hybridisation and wash conditions employed, the probes gave positive results with sequences containing up to three mismatches, as indicated in Table 5.3. None of the probes gave positive results with sequences containing greater than three mismatches. The complimentarity between the oligonucleotides and described methanogen species was also checked. Some of the probes had greater than the maximum tolerated mismatches with described species in their target groups, as indicated in Table 5.3. This meant that some of the probes would potentially not detect some described species in their target groups. The BLAST (Altschul *et al*, 1997) was used to check probe specificity and to search the GenBank database to identify sequences, which might give false-positive results. Target sequences potentially giving false-negative results and non-target sequences potentially giving false-positive results, with the probes are indicated in Table 5.3.

5.2.4 Detection of novel methanogens

The PCR primers used in this study are complimentary to highly conserved regions of the *mcrA* gene. Luton (1996) used these primers to amplify a fragment of the *mcrA* gene from 19 species of methanogen covering the whole methanogen phylogeny. PCR product was generated from three thermophilic species of *Methanococcus* using the same primers (P. Riley, personal communication). In this study, these same primers were used to generate PCR product from a further three species of methanogen. It is assumed therefore, that these primers will amplify the

Table 5.3 Probe specificity – sequences potentially giving false-negative or false positive results with the probes.

Probe	Maximum mismatches[a]	Target sequences with > maximum mismatches[b]	Non-target sequences with ≤ maximum mismatches[c]
mrtA-BAC1	0	Uncultured archaeon 83D (1)[d], Uncultured archaeon 95A (2)	
mcrA-BAC2	1		
mcrA-BAC9	2	*Methanobrevibacter ruminantium* (9), *Methanobrevibacter arboriphilicus* (3), Unidentified *Methanobacteriales* sp. (4), *Methanobacterium formicicum* (4), Uncultured methanogen RS-MCR45 (3), Landfill clone MS19 (3)	*Homo sapiens* BAC clone CTB-17C20 (2)
mcrA-MIC3	2	Landfill clones PL3 (5), OS63 (3)	*Homo sapiens*, v-raf murine sarcoma 3611 viral oncogene homolog 1 (1), *Homo sapiens* clone 23692 mRNA sequence (1), Human putative raf related protein (pks/a-raf) (1), Human mRNA for A-raf-1 oncogene (1), *Sus scrofa* mRNA for A-Raf-1 (1), Landfill clone PL28 (2)
mcrA-MIC4	0[e]		
mcrA-MIC5	3[f]		Landfill clone BSD63 (3)
mcrA-MIC8	0[e]		
mcrA-MIC10	1[f]	*Methanomicrobium mobile* (2), Landfill clones HL34 (2), OS77 (2), MS61 (2), HL81 (2), PL22 (2), PL240 (2), BSS46 (3)	
mcrA-MIC11/12	2[f]		*Homo sapiens* gastrin-releasing peptide receptor (GRPR) (2), *Homo sapiens* Xp22 BAC GS-321G17 (2), Uncultured *Archaea* 84C (2), 85A (2), Landfill clones OS55 (2), HL74 (2)
mcrA-SAR6	0[e]	*Methanosarcina siciliae* (1), *Methanosarcina vacuolata* (1), Uncultured methanogens RS-MCR36 (1), RS-ME42 (1), Landfill clone BSS21 (3)	
mcrA-ULM7	0[e]	Uncultured archaeon 85A (2)	

Key: The target and non-target columns list sequences that potentially give false-negative and false-positive results, respectively.

[a] The maximum number of mismatches tolerated with sequences giving positive results.

[b] Sequences in the target groups that contain greater than the maximum number of mismatches tolerated.

[c] Sequences outside of the target groups that contain less than or equal to the maximum number of mismatches tolerated.

[d] Number of mismatches with probe.

[e] All the reference sequences giving positive results contained zero mismatches.

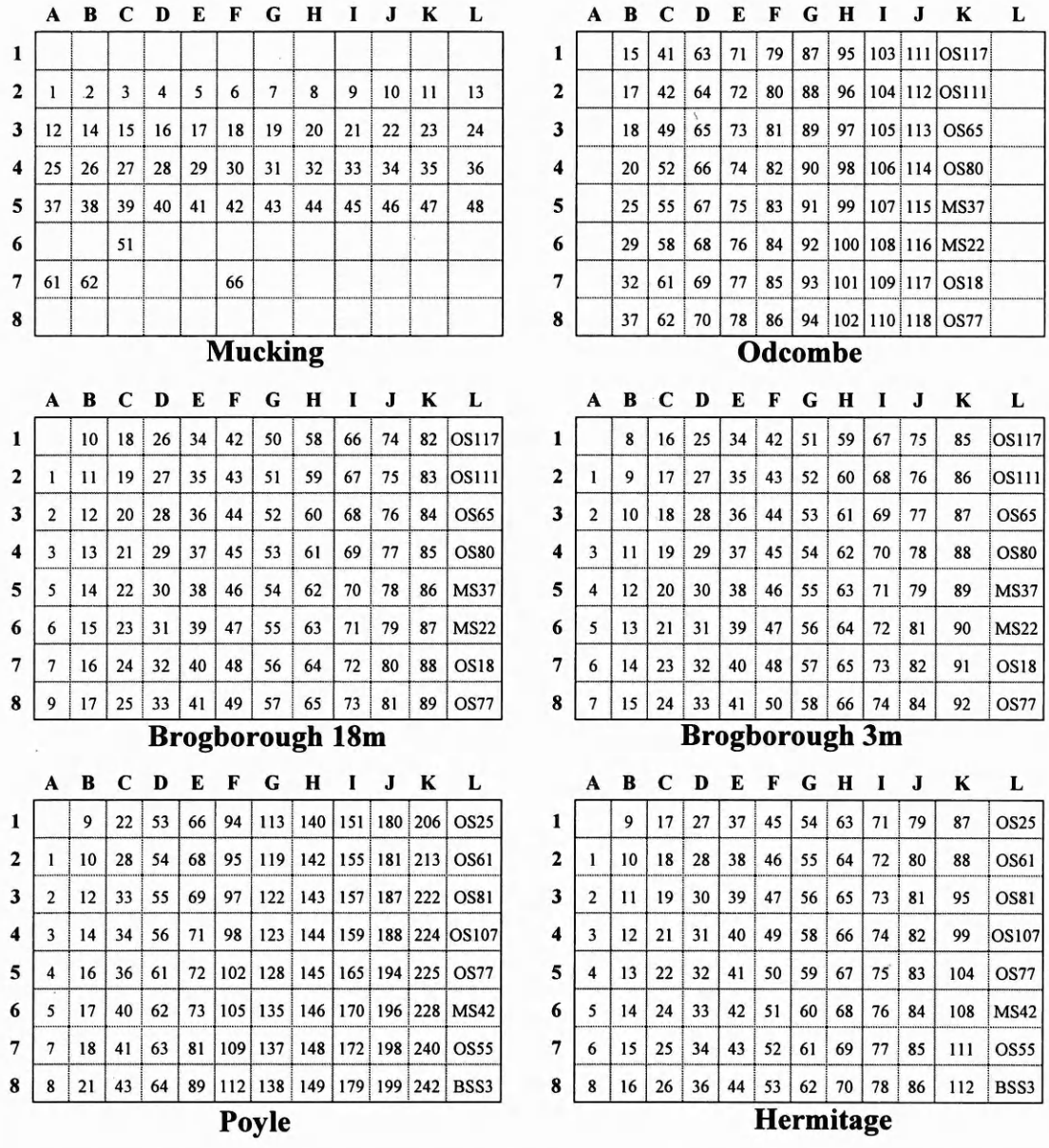
[f] Some sequences containing the maximum tolerated mismatches gave negative results.

mcrA gene from all methanogens. Cloned PCR products from the landfill samples, which were not detected by any of the *mcrA* probes, were sequenced so that new probes could be designed to cover these sequences. For example, three clones, PL53, BSD43 and BSD79, were not detected by probes 1 to 12. Sequencing and phylogenetic analysis of these clones showed that they were related to the obligate acetoclastic methanogen, *Methanosaeta concilii* (Figures 4.10 – 4.12). The *mcrA* sequences from these *Methanosaeta*–like clones were used to design a probe for this group, *mcrA*-SAE13 (Table 5.2). No non-target sequences with less than four mismatches were identified among the reference landfill clones or using the BLAST network service (Altschul *et al*, 1997).

5.2.5 Profiling methanogen populations in landfill

The probes were used to screen clone libraries of *mcrA* PCR products generated from six landfill samples. The cloned *mcrA* gene fragments were amplified by PCR and dot blotted onto nylon membranes (Figure 5.1). The dot blots were challenged with each of the probes. Plates 5.1 – 5.6 show the results of hybridising each of the probes and the PCR primer, *mcrA*-P3 reverse, to dot blots of clones from the Mucking, Odcombe, Brogborough 18m, Brogborough 3m, Poyle and Hermitage leachate samples. The results of the probing experiments are summarised in Table 5.4. Ninety-five percent of the clones assayed gave a positive result with one or more of the probes. Twenty-two clones were not detected by any of the probes (Table 5.4). Four clones from the Odcombe sample did not give a positive result with *mcrA*-P3 reverse. This may be because no DNA was spotted onto the membrane, or because the PCR product had degraded. The proportion of the clones in each population giving a positive result with each probe was plotted (Figure 5.2).

Figure 5.1 Key to dot blots of cloned *mcrA* PCR products from landfill samples.



	A	B	C	D	E	F	G	H	I	J	K	L
1												
2	1	2	3	4	5	6	7	8	9	10	11	13
3	12	14	15	16	17	18	19	20	21	22	23	24
4	25	26	27	28	29	30	31	32	33	34	35	36
5	37	38	39	40	41	42	43	44	45	46	47	48
6			51									
7	61	62				66						
8												

Mucking

	A	B	C	D	E	F	G	H	I	J	K	L
1		15	41	63	71	79	87	95	103	111	OS117	
2		17	42	64	72	80	88	96	104	112	OS111	
3		18	49	65	73	81	89	97	105	113	OS65	
4		20	52	66	74	82	90	98	106	114	OS80	
5		25	55	67	75	83	91	99	107	115	MS37	
6		29	58	68	76	84	92	100	108	116	MS22	
7		32	61	69	77	85	93	101	109	117	OS18	
8		37	62	70	78	86	94	102	110	118	OS77	

Odcombe

	A	B	C	D	E	F	G	H	I	J	K	L
1		10	18	26	34	42	50	58	66	74	82	OS117
2	1	11	19	27	35	43	51	59	67	75	83	OS111
3	2	12	20	28	36	44	52	60	68	76	84	OS65
4	3	13	21	29	37	45	53	61	69	77	85	OS80
5	5	14	22	30	38	46	54	62	70	78	86	MS37
6	6	15	23	31	39	47	55	63	71	79	87	MS22
7	7	16	24	32	40	48	56	64	72	80	88	OS18
8	9	17	25	33	41	49	57	65	73	81	89	OS77

Brogborough 18m

	A	B	C	D	E	F	G	H	I	J	K	L
1		8	16	25	34	42	51	59	67	75	85	OS117
2	1	9	17	27	35	43	52	60	68	76	86	OS111
3	2	10	18	28	36	44	53	61	69	77	87	OS65
4	3	11	19	29	37	45	54	62	70	78	88	OS80
5	4	12	20	30	38	46	55	63	71	79	89	MS37
6	5	13	21	31	39	47	56	64	72	81	90	MS22
7	6	14	23	32	40	48	57	65	73	82	91	OS18
8	7	15	24	33	41	50	58	66	74	84	92	OS77

Brogborough 3m

	A	B	C	D	E	F	G	H	I	J	K	L
1		9	22	53	66	94	113	140	151	180	206	OS25
2	1	10	28	54	68	95	119	142	155	181	213	OS61
3	2	12	33	55	69	97	122	143	157	187	222	OS81
4	3	14	34	56	71	98	123	144	159	188	224	OS107
5	4	16	36	61	72	102	128	145	165	194	225	OS77
6	5	17	40	62	73	105	135	146	170	196	228	MS42
7	7	18	41	63	81	109	137	148	172	198	240	OS55
8	8	21	43	64	89	112	138	149	179	199	242	BSS3

Poyle

	A	B	C	D	E	F	G	H	I	J	K	L
1		9	17	27	37	45	54	63	71	79	87	OS25
2	1	10	18	28	38	46	55	64	72	80	88	OS61
3	2	11	19	30	39	47	56	65	73	81	95	OS81
4	3	12	21	31	40	49	58	66	74	82	99	OS107
5	4	13	22	32	41	50	59	67	75	83	104	OS77
6	5	14	24	33	42	51	60	68	76	84	108	MS42
7	6	15	25	34	43	52	61	69	77	85	111	OS55
8	8	16	26	36	44	53	62	70	78	86	112	BSS3

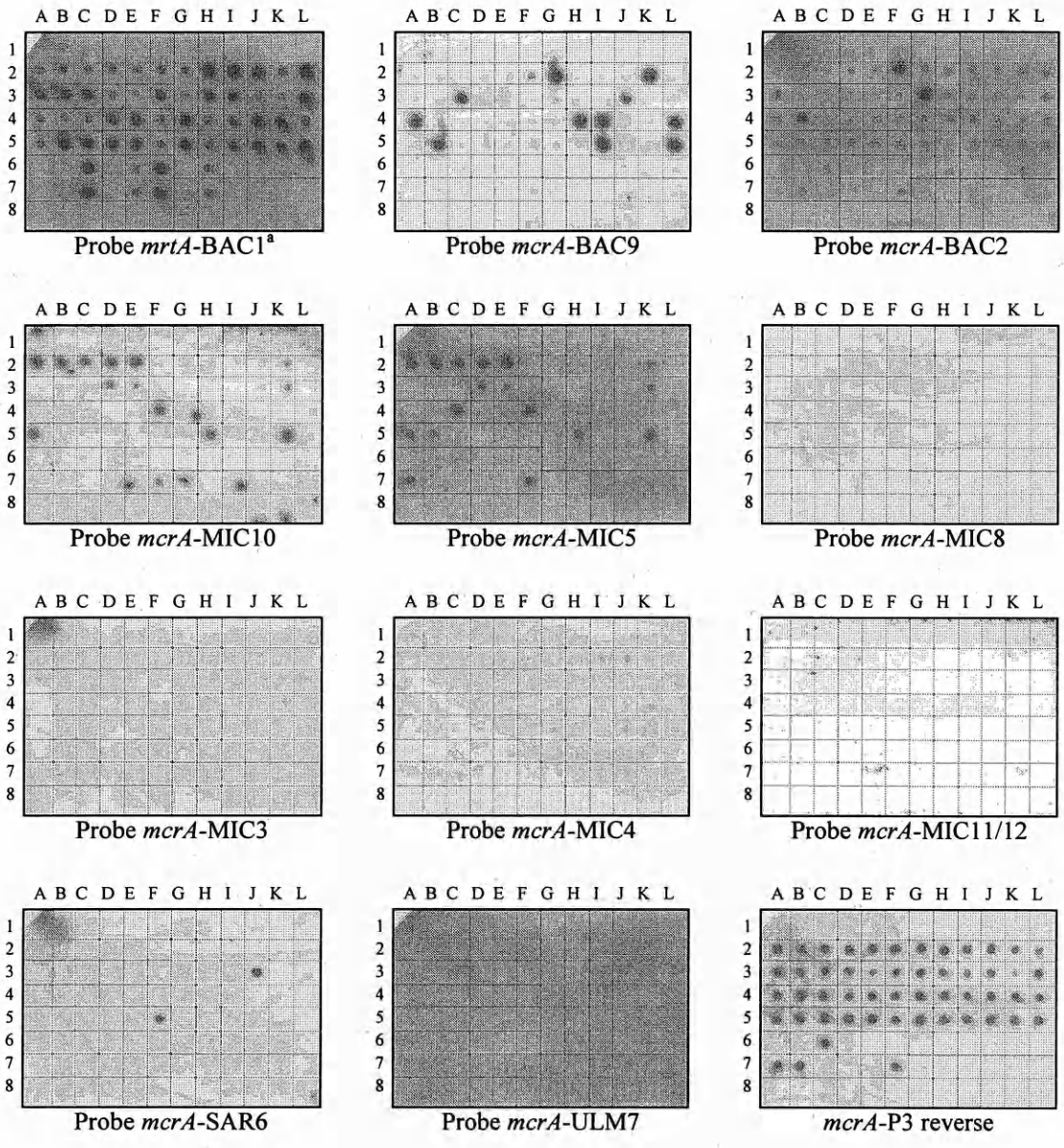
Hermitage

Key: *McrA* PCR products amplified and cloned from six landfill samples were blotted onto nylon membranes in an 8 × 12 grid, as described in section 2.13.2. The numbers in the boxes are the numbers of the clones from each landfill sample that were spotted onto the membrane at that position on the grid. The clones in column K of the Odcombe blot, and column L of the Brogborough 18m, Brogborough 3m, Poyle and Hermitage blots, were positive controls for each of the probes.

Positive controls

Probe	Clones
mrtA-BAC1	OS25, OS117
mcrA-BAC2	OS61, OS111
mcrA-BAC9	OS61, OS111
mcrA-MIC3	OS65, OS80, OS81, OS107
mcrA-MIC4	OS80, OS107
mcrA-MIC5	MS37, OS77
mcrA-MIC8	OS77
mcrA-MIC10	MS37, OS65, OS80, OS81, OS107
mcrA-MIC11/12	BSS3
mcrA-SAR6	MS22, MS42
mcrA-ULM7	OD18, OS55

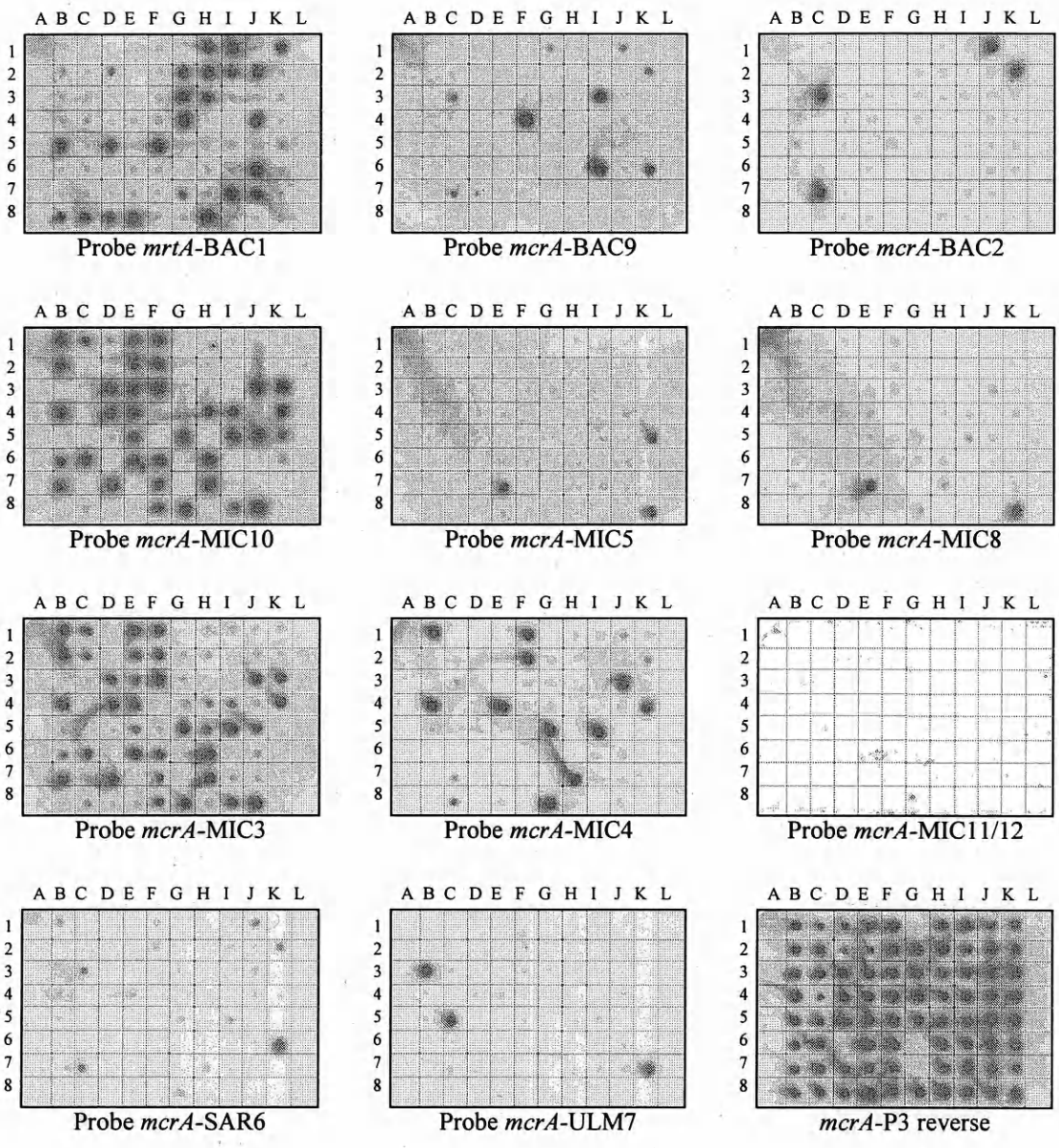
Plate 5.1 Results of hybridising *mcrA* probes to dot blots of cloned *mcrA* PCR products from the Mucking landfill enrichment culture.



Legend: The *mcrA* probes were hybridised to a dot blot of cloned *mcrA* PCR products from the Mucking landfill enrichment culture, as described in section 2.13. The primer *mcrA*-P3 reverse was hybridised to the blot as a positive control for each of the clones. Details of the clones spotted onto the membrane are given in Figure 5.1.

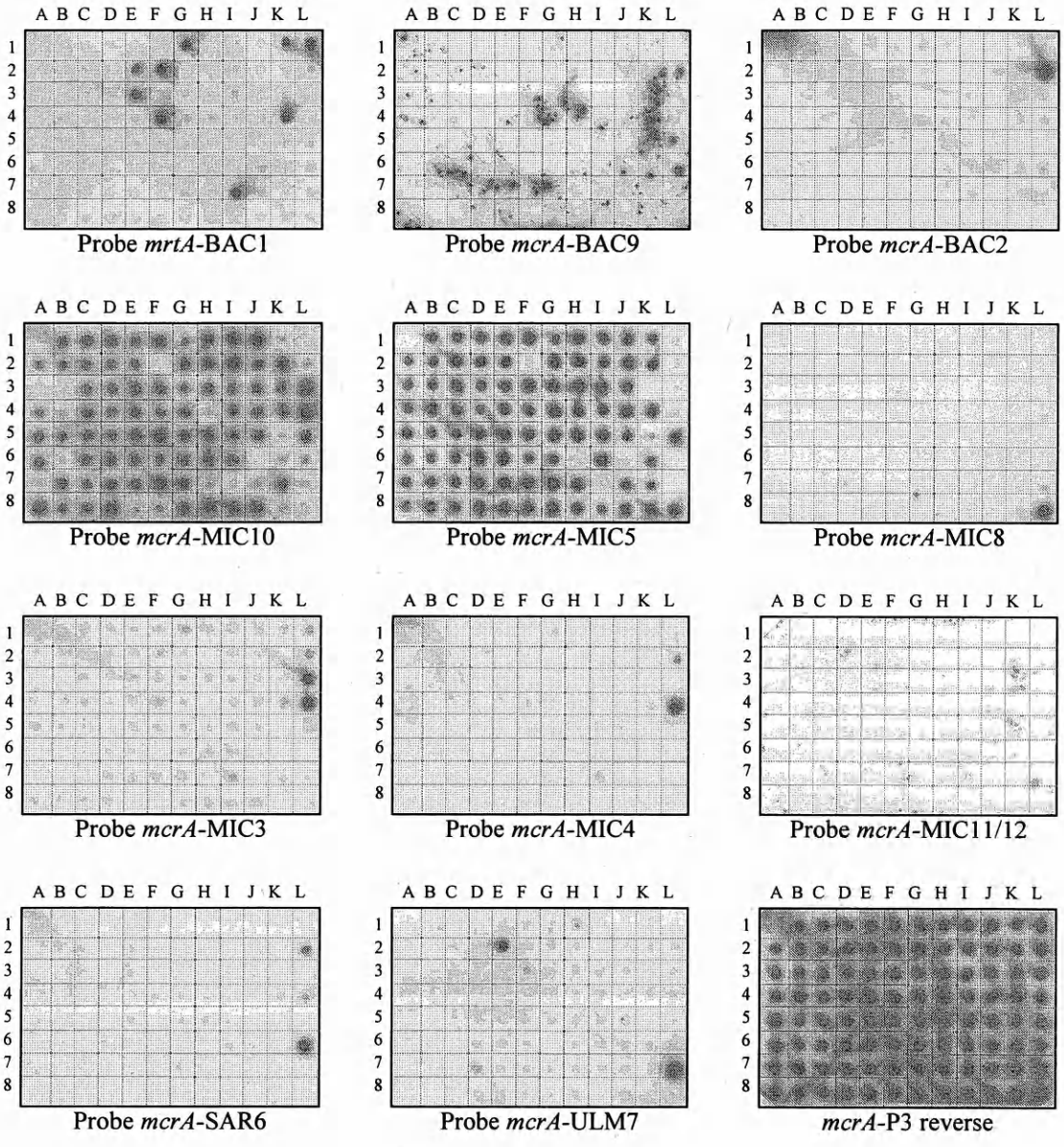
[a] The clones in rows 6 and 7 of this blot are arranged in a different order to that shown in Figure 5.1. The clones are arranged as follows: positions C6 and C7 = clone MS51; E6 and E7 = clone MS61; F6 and F7 = clone MS62; H6 and H7 = clone MS66.

Plate 5.2 Results of hybridising *mcrA* probes to dot blots of cloned *mcrA* PCR products from the Odcombe excavated refuse sample.



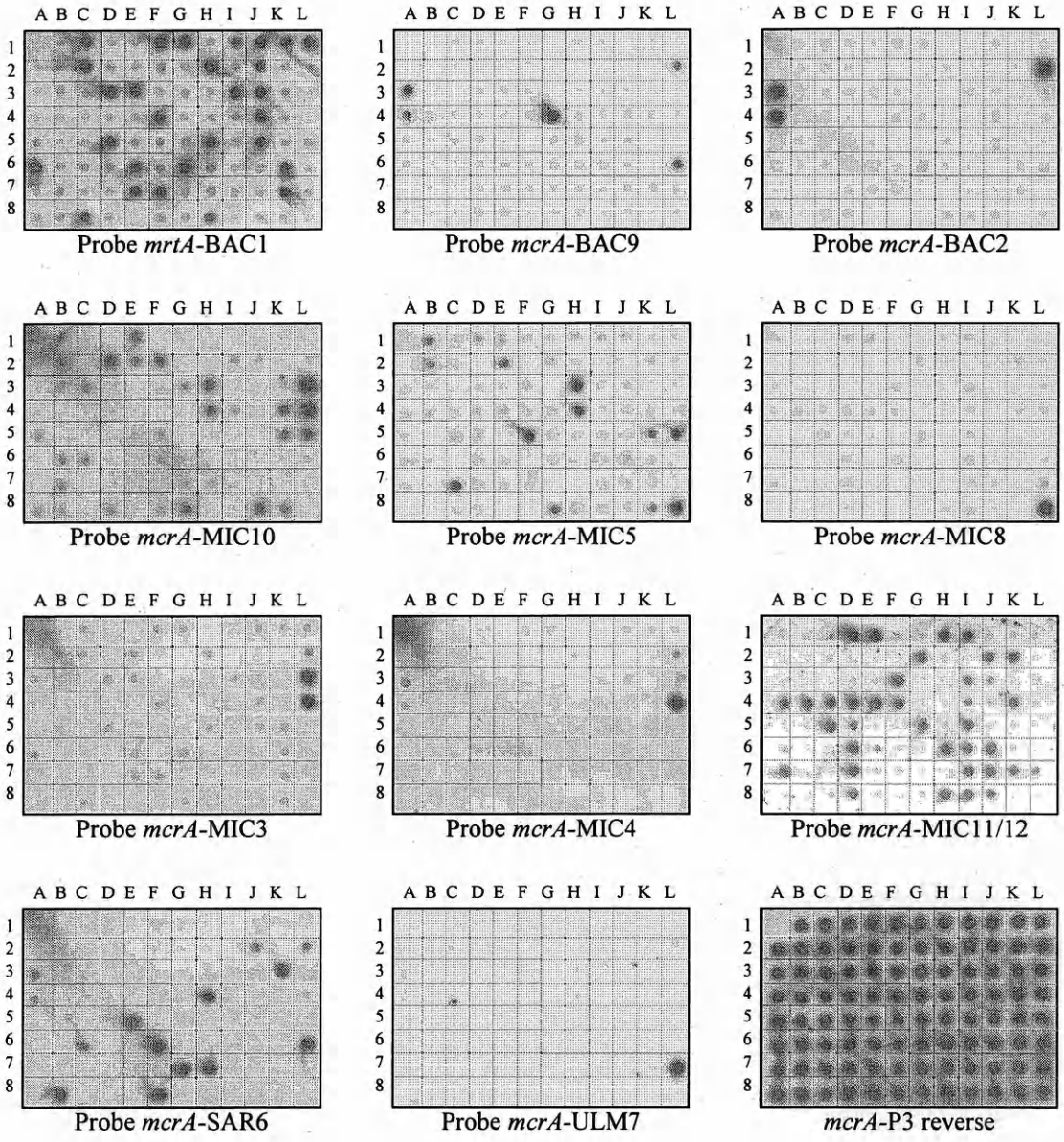
Legend: The *mcrA* probes were hybridised to a dot blot of cloned *mcrA* PCR products from the Odcombe excavated refuse sample, as described in section 2.13. The primer *mcrA*-P3 reverse was hybridised to the blot as a positive control for each of the clones. Details of the clones spotted onto the membrane are given in Figure 5.1. Clones known to give positive results with each of the probes were spotted onto the membrane in column K. Details of these positive controls are given in Figure 5.1.

Plate 5.3 Results of hybridising *mcrA* probes to dot blots of cloned *mcrA* PCR products from the Brogborough excavated refuse sample from 18m depth.



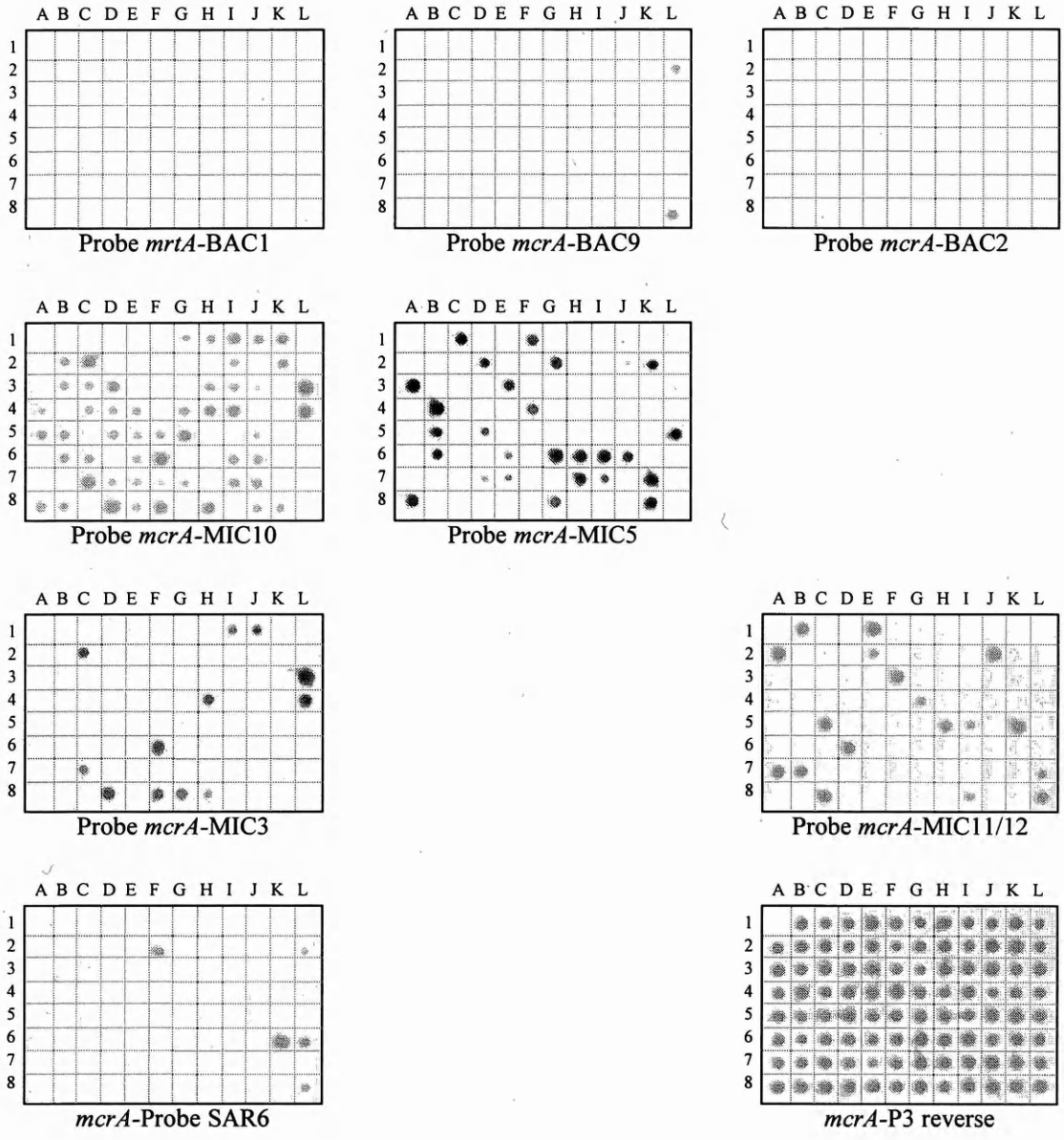
Legend: The *mcrA* probes were hybridised to a dot blot of cloned *mcrA* PCR products from the Brogborough excavated refuse sample from 18m depth, as described in section 2.13. The primer *mcrA*-P3 reverse was hybridised to the blot as a positive control for each of the clones. Details of the clones spotted onto the membrane are given in Figure 5.1. Clones known to give positive results with each of the probes were spotted on ton the membrane in column L. Details of these positive controls are given in Figure 5.1.

Plate 5.4 Results of hybridising *mcrA* probes to dot blots of cloned *mcrA* PCR products from the Brogborough excavated refuse sample from 3m depth.



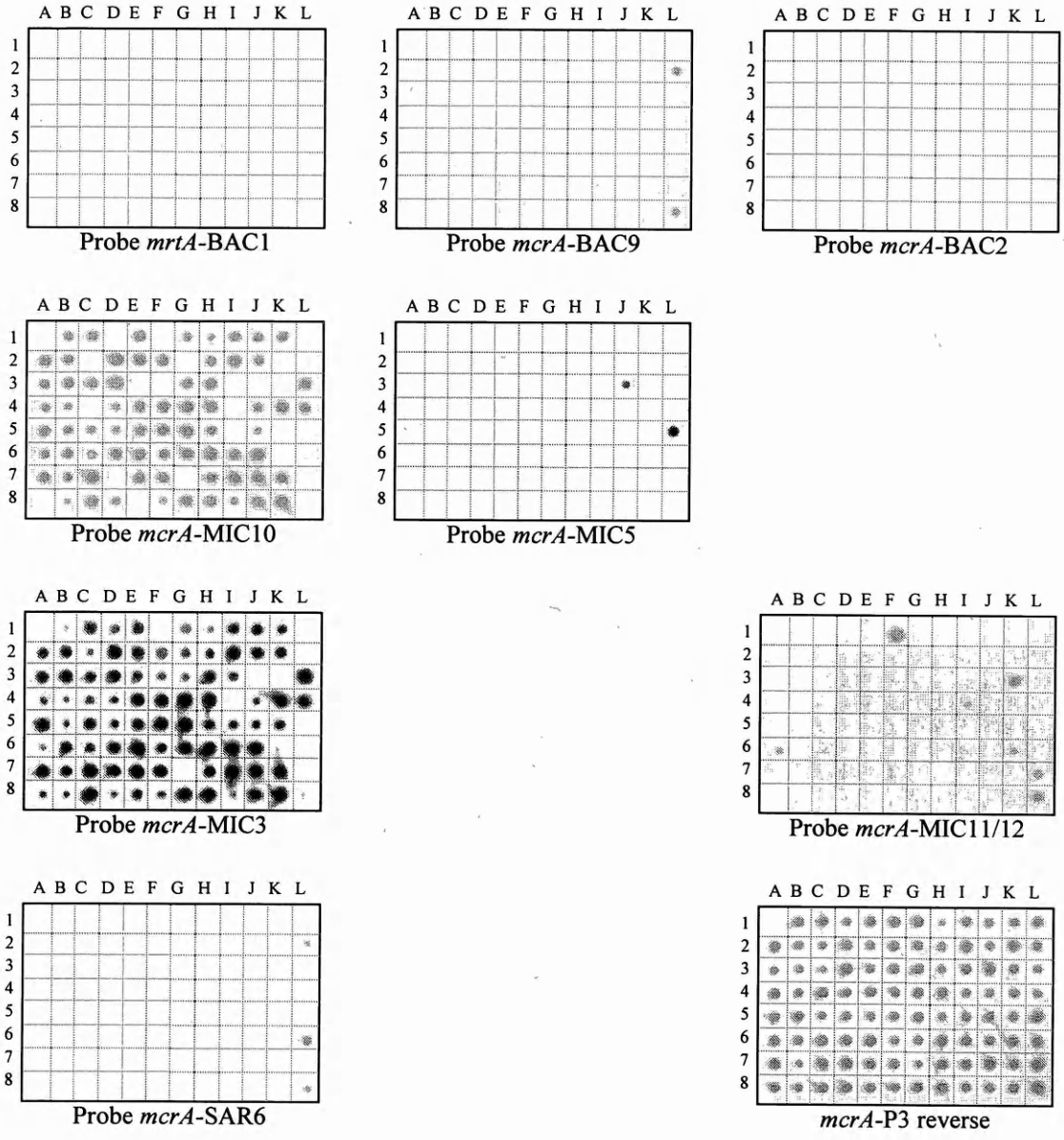
Legend: The *mcrA* probes were hybridised to a dot blot of cloned *mcrA* PCR products from the Brogborough excavated refuse sample from 3m depth, as described in section 2.13. The primer *mcrA*-P3 reverse was hybridised to the blot as a positive control for each of the clones. Details of the clones spotted onto the membrane are given in Figure 5.1. Clones known to give positive results with each of the probes were spotted on ton the membrane in column L. Details of these positive controls are given in Figure 5.1.

Plate 5.5 Results of hybridising *mcrA* probes to dot blots of cloned *mcrA* PCR products from the Poyle leachate sample.



Legend: The *mcrA* probes were hybridised to a dot blot of cloned *mcrA* PCR products from the Poyle landfill leachate sample, as described in section 2.13. The primer *mcrA*-P3 reverse was hybridised to the blot as a positive control for each of the clones. Details of the clones spotted onto the membrane are given in Figure 5.1. Clones known to give positive results with each of the probes were spotted onto the membrane in column L. Details of these positive controls are given in Figure 5.1. Probes MIC4, ULM7 and MIC8 were not hybridised to the membrane.

Plate 5.6 Results of hybridising *mcrA* probes to dot blots of cloned *mcrA* PCR products from the Hermitage leachate sample.



Legend: The *mcrA* probes were hybridised to a dot blot of cloned *mcrA* PCR products from the Hermitage leachate sample, as described in section 2.13. The primer *mcrA*-P3 reverse was hybridised to the blot as a positive control for each of the clones. Details of the clones spotted onto the membrane are given in Figure 5.1. Clones known to give positive results with each of the probes were spotted onto the membrane in column L. Details of these positive controls are given in Figure 5.1. Probes MIC4, ULM7 and MIC8 were not hybridised to the membrane.

Table 5.4 Summary of results from probing *mcrA* clones from six landfill samples with oligonucleotide probes.

Probes	Landfill samples						Total
	MS[a]	OS[b]	BSD[c]	BSS[d]	PL[e]	HL[f]	
mrtA-BAC1	22	22	1	20	0	0	66
mcrA-BAC2	3	3	0	1	0	0	7
mcrA-BAC9	11	6	0	2	0	0	20
mcrA-MIC3	0	35	0	0	10	81	126
mcrA-MIC4	0	10	0	0	-[g]	-[g]	10
mcrA-MIC5	16	1	82	10	24	1	134
mcrA-MIC8	0	1	0	0	-[g]	-[g]	1
mcrA-MIC10	13	34	77	10	49	69	252
mcrA-MIC11/12[h]	0	0	0	31	15	4	50
mcrA-SAR6	2	0	0	8	2	0	12
mcrA-ULM7	0	2	0	0	-[g]	-[g]	2
Not Detected[i]	0	1	2	11	7	1	22
mcrA-P3 reverse[j]	52	68	87	87	87	87	468
Total no. of clones probed	**52**	**72**	**87**	**87**	**87**	**87**	**472**

Key: Cloned, *mcrA* PCR products were dot blotted onto nylon membranes and the membranes were probed with each oligonucleotide. The numbers of clones giving positive results with each probe are indicated.

[a] Mucking, [b] Odcombe, [c] Brogborough 18 m, [d] Brogborough 3 m, [e] Poyle, [f] Hermitage leachate

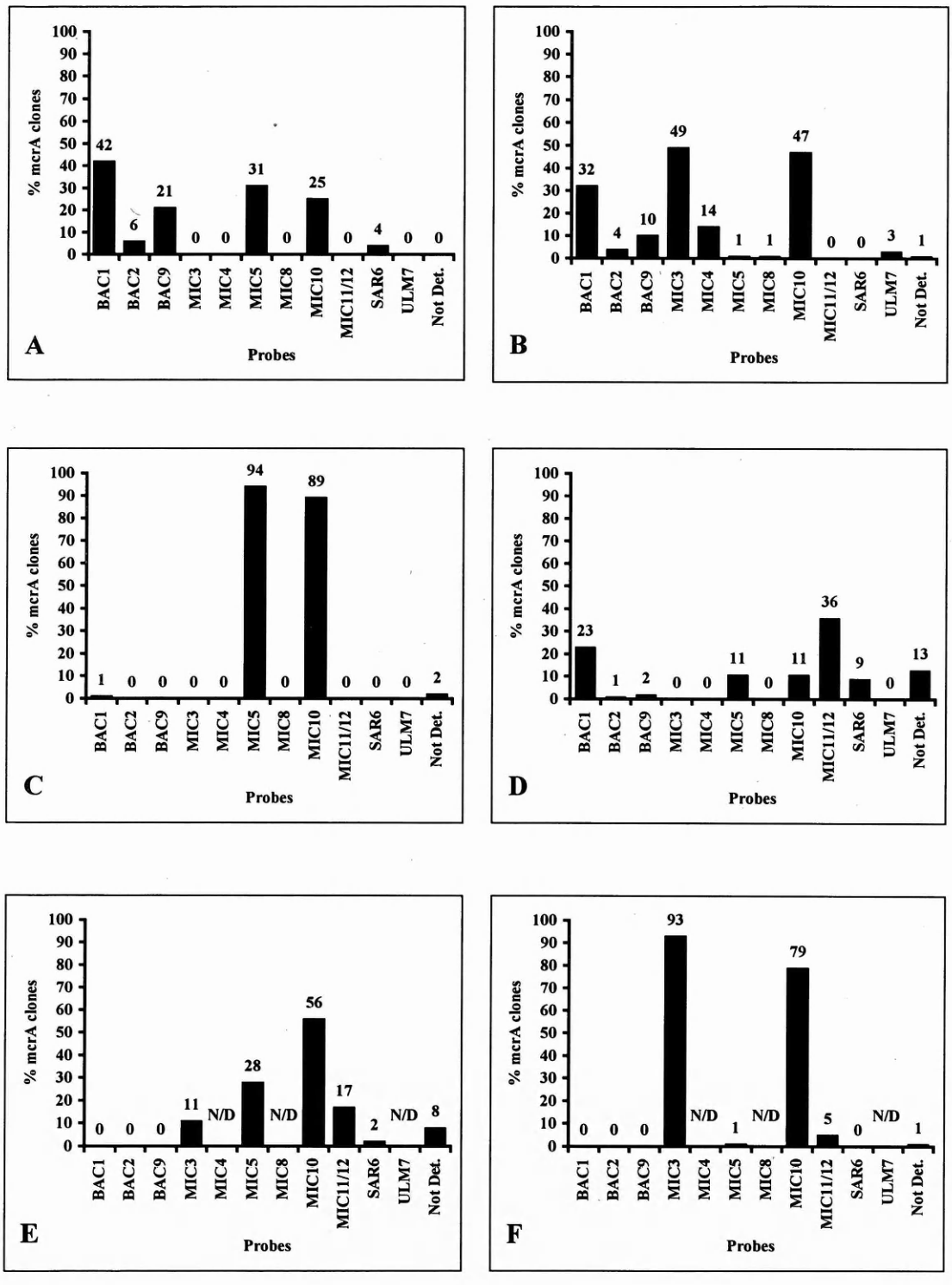
[g] Not determined

[h] Probes *mcrA*-MIC11 and *mcrA*-MIC12 were mixed in 1:16 molar ratio and used as a single probe.

[i] Numbers of clones not giving a positive result with any of the probes, not including *mcrA*-P3 reverse.

[j] The PCR primer, *mcrA*-P3 reverse, was used as a universal probe for all *mcrA/mrtA*.

Figure 5.2 Proportion of clones in six landfill samples giving a positive result with ten *mcrA* probes and one probe for *mrtA*.



Legend: Dot blots of *mcrA* PCR products cloned from six landfill samples were hybridised with oligonucleotide probes specific for different groups of methanogens. Landfill samples: (A) Mucking, (B) Odcombe, (C) Brogborough 18m, (D) Brogborough 3m, (E) Poyle and (F) Hermitage leachate. Not Det. = Clones not detected by any of the probes. N/D = not determined.

The order *Methanobacteriales* (probes *mrtA*-BAC1, *mcrA*-BAC2 and *mcrA*-BAC9) were abundant in the Mucking, Odcombe and Brogborough 3m samples. A single clone gave a positive result with probe *mrtA*-BAC1 in the Brogborough 18m sample, while no *Methanobacteriales* were detected in the Poyle or Hermitage samples. The high proportion of *Methanobacteriales* in the Mucking clone library was expected, since this library was generated from an enrichment culture grown in a *Methanobacterium* medium. Interestingly, in all the samples in which *Methanobacteriales* were detected, more clones gave a positive result with probe *mrtA*-BAC1, specific for *mrtA*, than with probes *mcrA*-BAC2 and *mcrA*-BAC9, which hybridise to *mcrA*.

The proportion of *Methanomicrobiales* in each sample, as measured by the percentage of clones hybridising with probes *mcrA*-MIC10 and *mcrA*-MIC11/12, ranged from 25% in the Mucking sample to 87% in the Brogborough 18m sample. The proportions of *Methanocorpusculaceae* (*mcrA*-MIC3), *Methanomicrobiaceae-Methanospirillaceae* (*mcrA*-MIC5) and 'unidentified landfill *Methanomicrobiales* cluster 1' (*mcrA*-MIC11/12) were different in each population. *Methanomicrobiaceae-Methanospirillaceae* predominated in the Mucking, Brogborough 18m and Poyle samples, while *Methanocorpusculaceae* predominated in the Odcombe and Hermitage samples, and the 'unidentified landfill *Methanomicrobiales* cluster 1' was the predominant *Methanomicrobiales* group in the Brogborough 3m sample (Figure 5.7). The Poyle sample was different in that probe *mcrA*-MIC10 gave a positive result with 49 clones, while probes *mcrA*-MIC3 and *mcrA*-MIC5 gave a positive result with 33 clones. Most of the clones causing this discrepancy belonged to 'unidentified landfill *Methanomicrobiales* clusters 2 and 3'.

The acetate and methylamine utilising *Methanosarcina* spp (*mcrA*-SAR6) were detected at low levels in the Mucking, Brogborough-3m and Poyle samples, but not at all in the other samples. This was surprising, given that it has been estimated that 60% of methane in anaerobic environments is generated from acetate (Ferry, 1992). Raskin *et al* (1994a) detected low levels of *Methanosarcina* in solid waste digesters fed with shredded municipal solid waste (MSW), using oligonucleotide probes for 16S rRNA. Probe *mcrA*-SAE13 was designed to detect the obligate acetotrophic methanogens of the family *Methanosaetaceae*. This probe was not hybridised to the dot blots. The results of the PCR-RFLP and phylogenetic analyses indicated that only two clones in the Brogborough-18m clone library and two clones in the Poyle clone library, belonged to the *Methanosaetaceae*.

Probes *mcrA*-MIC4, *mcrA*-MIC8 and *mcrA*-ULM7 were used against dot blots of Mucking, Odcombe and Brogborough clones, but not Poyle or Hermitage clones. These probes were based on a few sequences from the Odcombe clone library. As a result they were highly specific and only clones from the Odcombe clone library gave positive results with these probes. Phylogenetic analysis indicated that the target group of probe *mcrA*-ULM7, the 'unidentified landfill methanogens', contained a single clone from the Hermitage leachate sample. The PCR-RFLP indicated that three clones from the Poyle leachate sample might also belong to this group.

Twenty-two clones were not detected by any of the probes. The majority of these undetected clones were from the Brogborough-3m and Poyle samples. Several of these clones have been sequenced and subjected to phylogenetic analysis (Figures 4.1 – 4.12). Phylogenetic analysis showed that one of these clones, BSS12, was a *mrtA*

sequence distantly related to *mrtA* sequences from *Methanothermus fervidus* and *Methanosphaera stadtmanae* (Figures 4.4 – 4.6). Three clones were from the 'unidentified landfill *Methanomicrobiales* cluster 1' covered by probe *mcrA*-MIC11/12, and three clones were related to *Methanosaeta concilii*, as described above.

5.3 DISCUSSION

A set of oligonucleotide probes was designed for the *mcrA* gene to enable the rapid characterisation of methanogen populations in landfill. Oligonucleotide probes are excellent tools for describing natural communities. They can be used to provide qualitative and quantitative estimates of community structure (Hugenholtz and Pace, 1996). Probes can be tailor-made to bind targets with a wide range of specificities. In the case of the *mcrA* gene, probes could be designed to target all methanogens, individual orders, families, genera, species or strains. However, the design of such probes relies on sufficient sequence data being available. Previous studies have used sequence information from cultured methanogen species to design probes (Luton, 1996; Raskin *et al*, 1994b). Thus, they may not be free from cultivation limitation (Godon *et al*, 1997b). In this study, the sequence data for the *mcrA* gene, obtained from samples from landfill sites, was used to define phylogenetic groups, and subsequently to design oligonucleotide probes to target those groups. Thirteen probes were designed with different levels of specificity, from probes specific to individual clone sequences, up to a probe targeting several families. These probes allowed the description of almost the entire diversity of methanogen phylotypes identified in landfill.

5.3.1 Probe specificity

An assessment was made of the specificity of the probes by hybridising the probes to dot-blots of *mcrA* PCR products whose sequences were known, and by searching the GenBank database using BLAST. This showed that overall the probes were specific to their target groups. A few sequences were identified within the target groups, which either gave false-negative results, or might give false-negative results (Table 5.3). Many of these were published sequences from described or uncultured methanogens that showed affiliation to sequences from landfill. Positive results could be obtained with some of these sequences either by adjusting the sequence of the probes to be degenerate at the mismatched positions or by making the hybridisation conditions less stringent. A number of non-target sequences were identified that might give false-positive results with probes *mcrA*-BAC9, *mcrA*-MIC3 and *mcrA*-MIC11/12 (Table 5.3). Several of these sequences were from non-methanogens. However, these sequences do not pose a problem when the probes are used in combination with the methanogen-specific *mcrA* PCR primers. The specificity of these primers for methanogens has been confirmed by the failure to generate products from a range of non-methanogen DNA (Luton, 1996). In addition, a search of the GenBank database using the BLAST, failed to detect any non-target sequences likely to generate products with the reaction conditions employed for the PCR (results not shown). The clone HL74, from the cluster of 'unidentified landfill methanogens' (Figures 4.1 – 4.3, chapter 4) gave a false-positive reaction with probe *mcrA*-MIC11/12. Other sequences from this cluster, namely clone OS55 and the uncultured *Archaea* 84C and 85A, also contained just two mismatches with probe *mcrA*-MIC11/12. However, these sequences could be distinguished from other sequences giving positive reactions

with probe *mcrA*-MIC11/12, using the probe *mcrA*-ULM7, which was designed to target the 'unidentified landfill methanogens'.

5.3.2 Characterisation of methanogen communities

Hybridisation of the probes to dot blots containing between 52 and 87 clones from six of the landfill samples, demonstrated the utility of the probes for describing methanogen populations. This is most clearly illustrated in Figure 5.7, where the differences in population structure between the landfill samples can be seen. For example, the Brogborough 18m sample appears to be populated almost exclusively by the families *Methanomicrobiaceae* and *Methanospirillaceae*, whereas 93% of the population in Hermitage leachate sample are *Methanocorpusculaceae*. The other samples appear to contain populations that are more diverse. However, caution must be used when interpreting these semi-quantitative results due to the bias that may be introduced at each stage of the process, e.g. during the DNA extraction, the PCR and even the cloning step (Amann *et al*, 1995). The PCR-RFLP method also provided a view of the methanogen diversity and community structure in the landfill samples. However, the presence of 63 OTUs made interpretation of the results difficult. The group-specific probes supply the same information in a more easily interpreted form.

5.3.3 Previous studies employing methanogen-specific oligonucleotide probes

Oligonucleotide probes hybridising to the *mcrA* gene have been employed in previous studies. Luton (1996) used the *mcrA* sequences from 11 described species of methanogens to design species-specific probes. These probes were used to detect the target species in samples of landfill leachate. Positive reactions were obtained with the probes for *Methanoculleus bourgensis, Methanobacterium formicicum* and

Methanosarcina barkeri. Weak positive reactions were obtained with the probe for *Methanosphaera stadtmanae*. The probe for *M. bourgensis* cross-reacted with DNA from *Methanofollis liminatans* and *Methanospirillum hungatei*. Negative results were obtained with probes for *Methanothermobacter thermoautotrophicus, Methanobrevibacter ruminantium, Methanomicrobium mobile* and *Methanococcus voltae*. These results concur with the results obtained in this study using the group-specific *mcrA* probes, which also detected sequences related to *M. bourgensis, M. liminatans, M. hungatei, M. formicicum* and *M. barkeri* in landfill. Hales *et al* (1996) used a probe, which hybridised to the same conserved region of the *mcrA* gene as the *mcrA*-forward PCR primer used in this study, to screen clone libraries for clones containing *mcrA* PCR products.

Raskin *et al* (1994b) used sequences from described species to design group-specific probes that hybridised to the 16S rRNA of methanogens. The target groups of these probes included: *Methanobacteriaceae, Methanococcales, Methanomicrobiales, Methanosarcinales, Methanosarcina* and *Methanosaeta*. These probes have been used to quantify the different target-groups in anaerobic reactors (Raskin *et al*, 1994a). The abundances of the target-groups were expressed as percentages of the total 16S rRNA in the sample. The total 16S rRNA was quantified with a universal probe in combination with RNA standards. The relative rRNA abundance provides a reasonable measurement of the relative physiological activity of the respective population (Amann *et al*, 1995). This study found that *Methanomicrobiales* and *Methanosaeta* were the dominant methanogen groups in full-scale sewage sludge digesters (Raskin *et al*, 1994a). The same probes have been used to characterise the methanogen populations in the gastrointestinal tracts of domestic animals and landfill

(Lin *et al*, 1997; M. van Dyke, personal communication). M. van Dyke (personal communication) amplified 16S rDNA from landfill samples using *Archaea*-specific primers. They then hybridised the PCR products with the methanogen group-specific probes. Estimating diversity at the DNA level, rather than at the RNA level, theoretically provides a more accurate measurement of taxonomic group variability by potentially detecting slowly growing or dormant microorganisms present within the community (Moyer *et al*, 1994). M. van Dyke (personal communication) obtained intense hybridisation signals with the *Methanomicrobiales* probe with samples from two landfills. Intense hybridisation signals were also obtained for *Methanosarcinales* and *Methanosaeta* with one of the samples, but weaker signals with the other sample. The probe for *Methanosarcina* gave a weak signal with both samples, while the probe for *Methanobacteriales* gave a weak signal with one sample and no signal with the other sample. The apparent abundance of *Methanomicrobiales*, the low level of *Methanosarcina* and the low level or absence of *Methanobacteriales* is in agreement with the results of this study. However, the apparent abundance of *Methanosaeta* in one of the samples does not concur with the findings of this study.

The set of group-specific probes designed by Raskin *et al* (1994b) includes four probes to describe sub-divisions of the order *Methanosarcinales*, in addition to a probe covering the whole order. However, the set includes only a single probe to cover the order *Methanomicrobiales*. Given the diversity of phylotypes of *Methanomicrobiales* identified in the landfill samples (chapter 4), this set of probes would not adequately describe the methanogen populations in the landfill samples. For this study, probes were designed to target three sub-groups within the order *Methanomicrobiales*, which appeared to be abundant in landfill. These sub-groups

were the *Methanocorpusculaceae* (probe *mcrA*-MIC3), the *Methanomicrobiaceae+Methanospirillaceae* (probe *mcrA*-MIC5) and the 'unidentified landfill *Methanomicrobiales* cluster 1' (probe *mcrA*-MIC11/12). The use of probes specific for these three groups revealed clear differences in the methanogen populations of the landfill samples, as illustrated in Figure 5.7.

5.3.4 Potential applications of the group-specific *mcrA* probes

The group-specific probes for methanogens designed in this study have only been hybridised to dot blots of cloned *mcrA* PCR products. However, these probes could also be used in combination with the DGGE method to identify bands representing the different target-groups (Muyzer, 1998). Teske *et al* (1996) used a group-specific probe that hybridised to 16S rDNA to identify bands in a DGGE pattern, which were possibly derived from SRB. Any bands in a *mcrA* DGGE pattern that are not identified by the *mcrA* probes could be cut out of the gel, sequenced and identified by phylogenetic analysis. A variation of this procedure might use reverse transcriptase PCR (RT-PCR) to amplify the messenger RNA (mRNA) and so determine the relative *mcrA* mRNA abundance in each methanogen group, and hence the relative activity of each group. Oligonucleotide probes can also be hybridised directly to DNA extracted from environmental samples, or to whole-cells or particles from environmental samples fixed to a solid support. These procedures allow the quantification and localisation of the target-groups, and avoid PCR-introduced bias. The group-specific 16S rRNA probes for methanogens described above have been used in these procedures to quantify methanogen groups in anaerobic reactors (Raskin *et al*, 1994a), and to localise methanogens in sludge granules (Sekiguchi *et al*, 1999). However, a higher level of sensitivity would be required to detect probes hybridised to enzyme-

encoding genes compared to ribosomal RNA genes due to the lower abundance of specific mRNAs.

5.3.5 Summary

The set of group-specific oligonucleotide probes designed in this study, are a versatile tool that can be used with a variety of techniques to characterise the methanogen community in environmental samples. The probe target-groups were defined by phylogenetic analysis of *mcrA* sequences amplified from samples of landfills, and the design of the probes was based on these environmental sequences. Hence, these probes are more likely to hybridise to genes from uncultured methanogens compared to probes based solely on sequences from cultured species. The effectiveness of these probes for describing the methanogen community in landfill was demonstrated by screening clone libraries of cloned *mcrA* PCR products from six landfill samples. This provided a semi-quantitative measure of the methanogen community structure in these samples.

6 Detection of homoacetogenic bacteria by PCR and hybridisation with a functional gene probe

6.1 INTRODUCTION

Homoacetogens are a versatile group of strictly anaerobic *Bacteria*, able to grow on a variety of substrates. They catalyse the conversion of CO_2 + H_2 to acetate, and in some cases the reverse reaction (Hattori *et al*, 2000; Schnurer *et al*, 1996). Under certain conditions, such as low pH, low temperature, or in environments that are not strictly anoxic, homoacetogens can out-compete methanogens for hydrogen (Fey and Conrad, 2000; Schink, 1997). This is despite their lower affinity for hydrogen and lower energy yield compared to methanogens growing on hydrogen. There are no reports of homoacetogens in landfill and their significance in the landfill ecosystem has not been established (Palmisano and Barlaz, 1996). However, there is a need to determine the prevalence of homoacetogens in landfill, because of their position in the anaerobic food web, connecting the CO_2 + H_2 pool to the acetate pool, and their potential to compete with methanogens.

Lovell & Hui (1991) demonstrated the use of a functional group-specific DNA probe for the detection of homoacetogenic bacteria in environmental samples. The probe was based on the gene encoding the enzyme, formyl tetrahydrofolate synthetase (FTHFS). It was our intention to use this probe, in combination with PCR amplification of the FTHFS gene, to detect homoacetogens in landfill. The use of PCR would increase the sensitivity of detection and greatly facilitate the cloning and

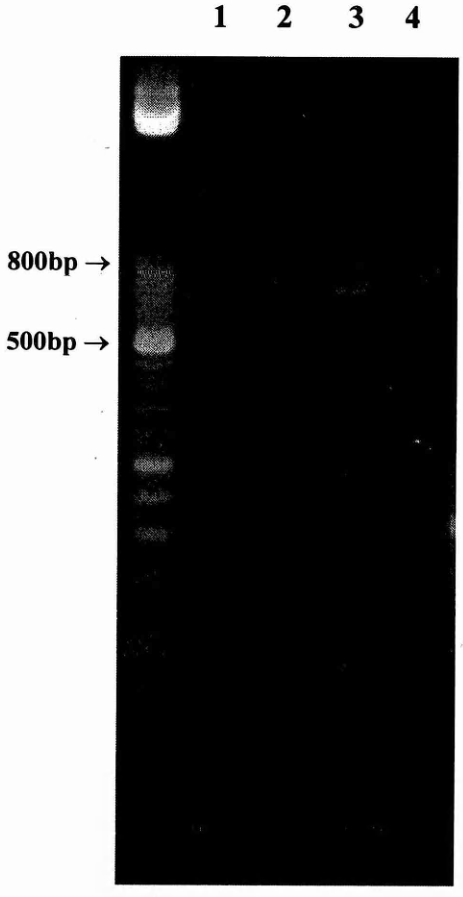
sequencing of FTHFS genes. Sequence data obtained from pure cultures and environmental samples could be used to improve the design of the primers. Ultimately, PCR amplification of the FTHFS gene from landfill samples followed by sequencing and phylogenetic analysis could be used to identify homoacetogens in landfill. This chapter describes the development of PCR amplification of the FTHFS gene and probing with the FTHFS gene probe.

6.2 RESULTS

6.2.1 Design and testing of PCR primers for the FTHFS gene and generation of PCR products from landfill

A range of PCR primers was designed for amplification of the FTHFS gene (Table 2.4). These primers were based on conserved regions of the FTHFS gene identified in alignments of the gene from a homoacetogen, *Moorella thermoacetica* and three non-acetogenic bacteria, *Clostridium acidiurici, Clostridium cylindrosporum* and *Streptococcus mutans*. The amino acid sequences were aligned using MEGALIGN (DNAStar) and scanned manually for conserved regions. The nucleotide sequences corresponding to the conserved regions were examined and primers designed with different levels of specificity. Primers were designed that would amplify all the aligned sequences. In addition, primers specific to FTHFS from *M. thermoacetica* were designed. The primers were tested in different combinations with DNA extracted from landfill. The majority of the primers failed to generate single-products of the predicted size. Single PCR products of the predicted size (682bp) were generated with the primers 328f17 and 992r18, from DNA extracted from two Brogborough landfill samples (Plate 6.1). No visible PCR product was generated

Plate 6.1 PCR products generated from landfill with primers for the FTHFS gene.



Legend: PCR products were generated using primers 328f17 and 992r18 (Table 2.4). Touchdown PCR was used with the annealing temperature dropping from 65°C to 60°C in the first five cycles followed by a further 20 cycles with an annealing temperature of 60°C. Lanes: 1) Negative control, no template; 2) Brogborough 3m sample; 3) Brogborough 18m sample; 4) Poyle leachate sample. A 50bp DNA ladder was run in the left-hand lane as a molecular size marker.

from the Poyle landfill leachate sample with these primers. Touchdown PCR was used to improve the specificity of the amplification with this primer pair.

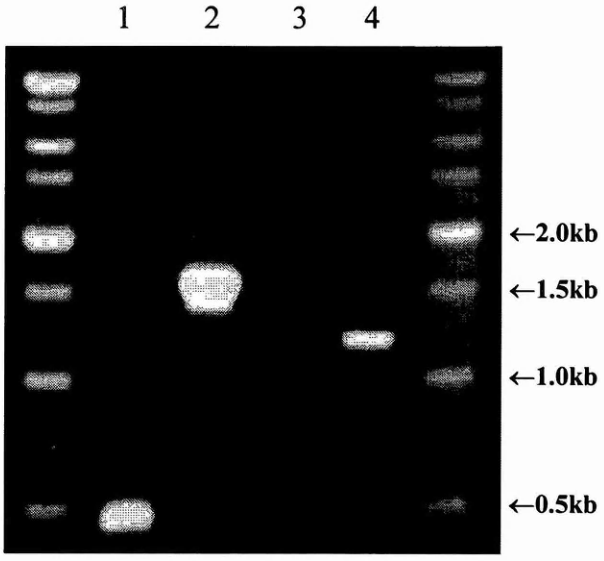
6.2.2 Amplification and labelling of an FTHFS gene probe for homoacetogens

To generate the functional group-specific DNA probe for acetogens described by Lovell and Hui (1991), primers were designed to amplify a 1.387kb fragment of the FTHFS gene from *M. thermoacetica* (Table 2.4). The amplified fragment corresponded to the 1.382kb *Hinc* II – *Kpn* I fragment used by Lovell and Hui (1991). A PCR product was generated from DNA extracted from a culture of *M. thermoacetica* strain DSM 521, and labelled with digoxigenin (Plate 6.2), as described in sections 2.16 – 2.19. The labelled PCR product was purified by excising the band from an agarose gel to remove non-specific by-products that would reduce the specificity of the probe.

6.2.3 Amplification of the FTHFS gene, blotting of PCR products and hybridisation with FTHFS gene probe

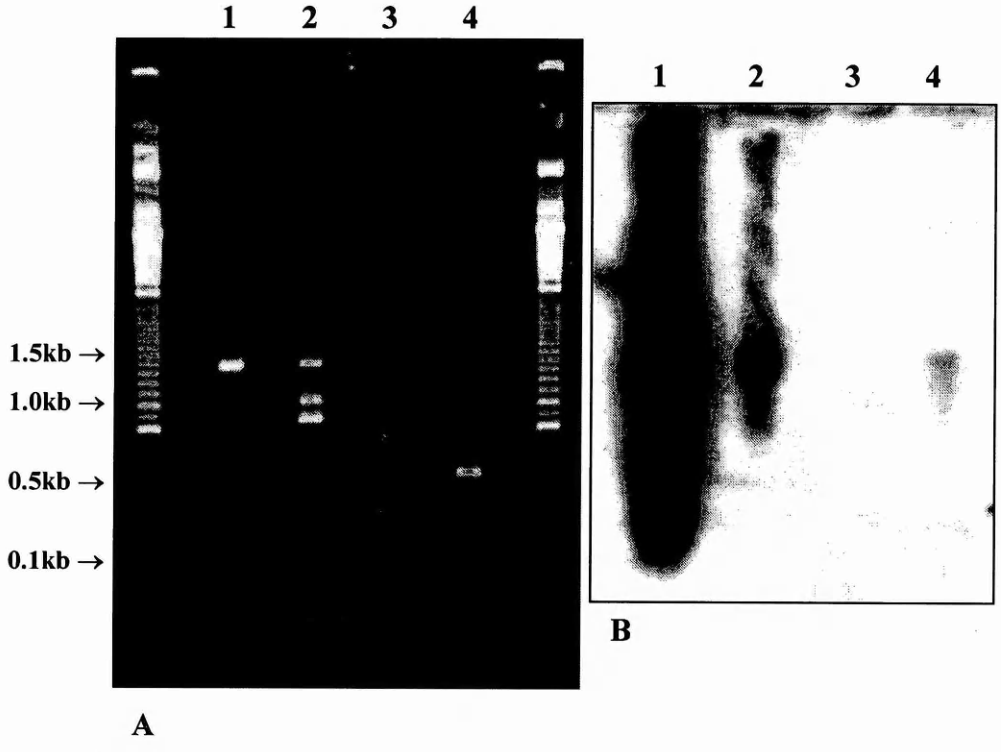
Hybridisation with the FTHFS gene probe was used to confirm that products amplified with the PCR primers were FTHFS. PCR products were generated from *M. thermoacetica*, *Acetobacterium wieringae*, *Sporomusa acidovorans* and the Brogborough 3m landfill sample, using primers 109f17 and 1465r17 (Plate 6.3a). All of the amplification reactions generated non-specific products either in the form of a smear or discrete bands that were not the predicted size, 1.356kb. The PCR products were run on an agarose gel, then blotted onto a nylon membrane, as described in section 2.19. The digoxigenin-labelled FTHFS gene probe was hybridised to the membrane and detected by chemiluminescent detection (Plate 6.3b). The FTHFS gene

Plate 6.2 Amplification and labelling of a functional group-specific DNA probe for homoacetogenic bacteria.



Legend: PCR product generated from *M. thermoacetica* DNA using primers 14f19 and 1400r18 (Table 2.4) was used as the template in a PCR reaction that incorporated digoxigenin-11-dUTP into the product. Lanes: 1) Positive control reaction for PCR DIG Probe Synthesis Kit; 2) Digoxigenin-labelled FTHFS gene PCR product; 3) Negative control reaction, no template; 4) FTHFS gene PCR product. The digoxigenin-labelled PCR product in lane 2 appears larger than the unlabelled product in lane 4 due to multiple incorporation of digoxigenin-11-dUTP. A 500bp DNA ladder was run in the outside lanes as a molecular size marker.

Plate 6.3 Amplification of the FTHFS gene, blotting and hybridisation with FTHFS gene probe.



Legend: PCR products were generated from pure cultures of three homoacetogenic bacteria and from the Brogborough 3m landfill sample using primers 109f17 and 1465r17 (Table 2.4). The PCR products were run on an agarose gel (A), then blotted onto a nylon membrane. The digoxigenin-labelled FTHFS probe was hybridised to the membrane and detected by chemiluminescent detection (B). Lanes: 1) *M. thermoacetica*; 2) *A. wieringae*; 3) *S. acidovorans*; 4) Brogborough 3m landfill sample. A 100bp DNA ladder was run in the outside lanes of the agarose gel (A) as a molecular size marker.

probe gave a very strong signal with a wide range of amplification products from *M. thermoacetica*. The probe also gave a strong signal with bands of the predicted size from *A. wieringae*. The probe did not hybridise to any bands from *S. acidovorans*. Clear PCR products of the predicted size were not visible on the agarose gel either for *S. acidovorans*. The probe did hybridise weakly to amplification products of the predicted size from the Brogborough 3m landfill sample, even though no clear bands of that size were visible on the agarose gel.

6.3 DISCUSSION

"There is hardly any transformation process in an anoxic environment in which homoacetogens do not participate or with which they do not compete" (Schink, 1994). Homoacetogens as a group are able to participate in the fermentation of a wide variety of compounds including hydrogen and carbon dioxide (Drake *et al*, 1997). Due to this metabolic versatility it is not surprising that homoacetogens have been isolated from a diverse range of anaerobic habitats (Drake, 1994). Although there are no reports of homoacetogens isolated or detected in landfill, they must certainly be present. Previous studies of acetogenic bacteria or acetogenic activity in connection with landfill, have focused on the syntrophic fatty-acid oxidising acetogens, or have not distinguished between this group and homoacetogens (Cooke *et al*, 1999; James *et al*, 1998; Lay *et al*, 1998a; Qian & Barlaz, 1996).

6.3.1 The role of homoacetogens in landfill

Our knowledge of the role of homoacetogens in landfill is superficial. Figure 1.6 (chapter 1) shows the homoacetogens as being involved only in the conversion of CO_2

+ H_2 to acetate. It is likely that homoacetogens are also involved in the fermentation of sugars, alcohols, glycerol and a range of other compounds in landfill. In addition to hydrogen and carbon dioxide, homoacetogens can utilise other methanogenic substrates such as formate, methanol, methylamines and even acetate (Schink, 1994). Some strains have been shown to carry out a reverse homoacetogenic reaction, cleaving acetate to form H_2 and CO_2 in syntrophic association with a hydrogen-utilising species (Hattori *et al*, 2000; Schnurer *et al*, 1996). Homoacetogens compete with other groups of microorganisms for all of the above mentioned substrates. However, they appear to be, in every case, inferior to the respective specialists (Schink, 1994). Homoacetogens grow slower on sugars than classical fermenters such as *Clostridium butyricum* or *Escherichia coli*, and they have a lower affinity for hydrogen than methanogens or SRB (Schink, 1994). Hence, in ecosystems where hydrogenotrophic methanogenesis or sulphate-reduction predominate, the hydrogen partial pressure is maintained at a level too low for homoacetogenesis by the methanogens or SRB (LeVan *et al*, 1998).

Homoacetogens are able to outcompete other groups under certain conditions. In a mildly acidic lake sediment, the entire electron flux from biomass to methane was shown to go through the acetate pool, due to the specific inhibition of hydrogenotrophic methanogens (Schink, 1994). Under these conditions, homoacetogens would take over the function of hydrogenotrophic methanogens in the utilisation of hydrogen and one-carbon compounds. At low temperatures (<20°C), in anoxic paddy soils, tundra wetland soils and lake sediments, homoacetogenesis from H_2 + CO_2 by psychrophilic acetogens appears to be the predominant hydrogen-consuming process (Diekert, 1992; Kotsyurbenko *et al*, 1996). Enrichment cultures

inoculated with freshwater sediment samples and with glycolate as the growth substrate lead to the isolation of a homoacetogenic co-culture before a methanogenic co-culture could be established (Friedrich *et al*, 1991). Homoacetogens may also have the advantage over methanogens in anaerobic environments that are subject to periodic oxygenation because they are less sensitive to oxygen-exposure than methanogens (Schink, 1994). Conditions such as low pH and periodic infiltration of oxygen are known to occur in landfills (Anon, 1988; Caine *et al*, 1999). Under these circumstances, homoacetogens may be important in the degradative process. Homoacetogens may also play a more significant role in the early stages of degradation, before the establishment of methanogenesis.

6.3.2 Detection of homoacetogens

Like other groups of microorganisms, many homoacetogens extant in nature are probably unknown because of our inability to cultivate these species. Culture-based methods have been used to specifically isolate or enumerate acetogens in environmental samples. For example, the colourimetric most-probable-number assay used by Harriott and Frazier (1997), or the enrichment of homoacetogens using aromatic substrates carried out by Peters and Conrad (1995). As demonstrated in the previous chapters for methanogens, molecular methods provide the ability to detect and identify specific groups of microorganisms in the environment without the need to cultivate. However, unlike the methanogens, the homoacetogens do not form a coherent phylogenetic group (Tanner & Woese, 1994). There are currently around 17 genera containing homoacetogenic species. Many of these genera also contain non-acetogenic species. Although, this classification is, in part historical, and not based on phylogenetic relationships, it is still indicative of the diverse phylogeny of the

homoacetogens. Since the homoacetogens are phylogenetically diverse the development of a probe or PCR primers based on conserved 16S rRNA sequences that could be used to detect many or all acetogens simultaneously would be difficult (Lovell and Hui, 1991). A solution to this problem would be to develop a DNA probe or PCR primers based on the signature property of homoacetogens, the production of acetate from C_1 compounds. This would allow the homoacetogens to be treated like the methanogens, as a functional group.

Group-specific probe for homoacetogens

Lovell and Hui (1991) developed a functional group-specific DNA probe for the homoacetogens based on the FTHFS gene from *Moorella thermoacetica*. This gene encodes formyltetrahydrofolate synthetase, an essential enzyme in the acetyl-CoA pathway. This enzyme is structurally and catalytically very similar among the known acetogens (Lovell and Hui, 1991). FTHFS activity is readily detected in acetogens, but is absent from many other *Bacteria* (Lovell and Hui, 1991; Whitehead *et al*, 1988). A search of the GenBank database using the BLAST identified enzymes from a range of *Bacteria* and *Eucarya* showing varying degrees of amino acid sequence homology to FTHFS from *M. thermoacetica* (results not shown). At the DNA level, the FTHFS gene from a non-acetogen, *Clostridium acidiurici*, has only 61% nucleotide sequence homology and no significant stretches of sequence identity, with the FTHFS gene from *M. thermoacetica* (Lovell and Hui, 1991). Lovell and Hui (1991) designed their homoacetogen-specific DNA probe based on this information, and tested it with DNA from a range of homoacetogens and non-acetogens.

PCR of the FTHFS gene

In this study, PCR primers were designed to amplify the FTHFS gene. PCR methods are substantially more sensitive, less expensive and less time-consuming than most alternative procedures. However, PCR relies on the availability of sequence data for the design of suitable primers. The only FTHFS gene sequence available from a homoacetogen, was that from *M. thermoacetica*. This sequence was aligned with sequences from three non-acetogenic *Bacteria*, and the alignment was used to design primers with different levels of specificity. Two primers based on highly conserved nucleotide regions in the four FTHFS sequences were successful in amplifying a product of the predicted size from DNA extracted from two landfill samples. However, because these primers were based on regions of highly conserved sequence from both a homoacetogen and three non-acetogens, it is possible that the amplified products were from non-acetogens. Primers could be designed that would specifically amplify the FTHFS gene from *M. thermoacetica* and not the three non-acetogens. However, it is likely that such primers would fail to amplify the FTHFS gene from other homoacetogens. More FTHFS nucleotide sequences from homoacetogens are required to determine if it is possible to design homoacetogen-specific PCR primers.

Generation of the FTHFS gene probe and hybridisation with PCR products

To overcome the problem of the unknown specificity of the PCR primers, it was proposed to use the FTHFS gene probe to identify PCR products generated from homoacetogens. The probe was generated by PCR amplification of a 1.387kb fragment of the FTHFS gene from *M. thermoacetica*, and labelled with digoxigenin (DIG) by multiple incorporation of DIG-dUTP during PCR. This produced a highly sensitive probe. The DIG-PCR-labelled probe was tested with PCR products

generated from three homoacetogens, *M. thermoacetica, A. wieringae* and *S. acidovorans* as well as PCR product generated from the Brogborough 3m landfill sample. The PCR products were generated with highly degenerate 17-mer primers. These primers were designed with a high level of degeneracy in the third base position to attempt to encompass all possible sequence combinations, and hence broaden the specificity of the primers. Hybridisation of the probe with the PCR products gave very strong hybridisation signals with a wide molecular size range from *M. thermoacetica.* This could indicate both that the PCR used to generate the blotted PCR product and the PCR used to generate the probe had produced non-specific amplification products. The specificity of the probe could be improved by cloning the PCR product, and then generating the DIG-labelled PCR product from the cloned gene. This would avoid the problem of contamination of the probe with non-FTHFS DNA from *M. thermoacetica.* The hybridisation also appeared to indicate that FTHFS had been amplified from *A. wieringae* and the landfill sample, but not from *S. acidovorans.* Unfortunately, the hybridisation was performed only once and the specificity of the probe under the hybridisation and wash conditions employed is not known. Lovell and Hui (1991) showed that the level of stringency affected strongly the hybridisation of the probe to DNA from homoacetogens not closely related to *M. thermoacetica.*

6.3.3 Summary

- PCR primers were designed for the FTHFS gene and used to amplify a product of the predicted size from DNA extracted from two landfill samples.
- A highly sensitive probe for the FTHFS gene from homoacetogens was generated by PCR.

- Positive results were obtained when this probe was hybridised to PCR products amplified from the homoacetogens *M. thermoacetica* and *A. wieringae*, but not *S. acidovorans*.
- A positive result was also obtained when the probe was hybridised to PCR product amplified from a landfill sample.
- More FTHFS sequences from homoacetogens are needed to improve the design of the PCR primers.
- Further work is required to optimise the hybridisation and wash conditions for the probe, and to ensure that the probe generated by PCR is specific for FTHFS.

The work presented in this chapter represents the first step in the development of molecular methods for the investigation of the homoacetogen community in landfill and other environments. The ultimate aim would be to use the FTHFS gene as a functional marker to investigate the distribution, diversity, composition, abundance and activity of homoacetogens in landfill, in the same way that the *mcrA* gene has been used to describe methanogen communities in landfill. The potential to achieve this aim has been facilitated greatly by the recent publication from Leaphart & Lovell (2001). They reported the PCR amplification of a 1.102kb fragment of the FTHFS gene from both known acetogens and other FTHFS-producing organisms. Phylogenetic analysis of sequences from the amplified products showed that FTHFS sequences from homoacetogens formed a monophyletic cluster that did not contain sequences from non-homoacetogens.

7 GENERAL DISCUSSION

Why study landfill microbiology?

Landfill is the principal means of disposal for the majority of municipal, commercial and industrial solid-waste in the United Kingdom. The action of microorganisms in breaking down the organic fraction of waste in landfills results in the production of landfill gas and leachate. Landfill gas consists predominantly of methane and carbon dioxide, both of which are 'greenhouse' gases. Landfills account for 31% of the European Communities methane emissions (Anon, 1996). Hence, there is a need to control the emission of gases from landfills. In addition, the methane in landfill gas represents a potentially exploitable energy source. At the end of 1998 there were 107 projects in the UK generating 200 megawatts of power from landfill gas (Anon, 2000f). Leachate, which contains compounds leached from the waste, has the potential to pollute ground and surface water if it escapes from a landfill. It is necessary therefore to control the production of leachate and treat it before disposal. A better understanding of the microbial communities and processes responsible for biodegradation in landfills could lead to improved control of landfill gas and leachate production (Lawson, 1989b). Furthermore, optimisation of microbial activity in decomposing refuse could produce a number of benefits including: increased total methane production and rate of production; minimisation of nuisance compounds such as volatile fatty acids or potentially toxic substrates in landfill gas and leachate; shorter time to achieve waste stabilisation; and more rapid and greater reduction in waste volume leading to more space for fresh waste (Palmisano and Barlaz, 1996).

The decomposition process in landfill

The decomposition process in landfill can be divided into several stages each mediated by a different functional-group of microorganisms (Anon, 1988; Anon, 1995b; Barlaz, 1996). The first stage of this process involves the degradation of polymers, principally cellulose, by aerobic hydrolytic microorganisms and after the oxygen has been depleted, by anaerobic hydrolytic microorganisms. In the second stage, anaerobic fermentative *Bacteria* convert the hydrolysis products to a variety of oligomeric carbon compounds. The third stage is characterised by the conversion of fatty acids to acetate, carbon dioxide and hydrogen by syntrophic acetogens. In the fourth and final stage, the methanogenic *Archaea* convert $H_2 + CO_2$, acetate and a few other simple carbon compounds to methane. Other groups of *Bacteria* such as sulphate-reducing bacteria and homoacetogens may also be involved in the degradation process, and may compete with methanogens for hydrogen.

Methanogens and molecular methods

The direct involvement of methanogens in methane generation has lead to more interest in this group compared to the other groups described above. Yet relatively little is known about the diversity and composition of the methanogen community in landfills. This is due in part to the difficulties encountered when using traditional culture-based methods to investigate these slow-growing, fastidious, obligate anaerobes. Molecular methods, such as PCR and hybridisation with DNA probes, which target the nucleic acids in cells directly, provide a means of investigating microbial communities in the environment without the need to cultivate. In addition, the information contained in the genetic material allows the identity of organisms in the environment to be rapidly and accurately determined. Furthermore, this

information can be used to make predictions about the physiology and growth-requirements of unknown organisms, thus allowing them to be cultured (Hugenholtz and Pace, 1996; Stahl, 1997).

PCR

In this study, a range of molecular techniques was employed to investigate the diversity and structure of the methanogen community in landfills. The basis of these techniques was the use of PCR to amplify a gene believed to be unique to methanogens. PCR allows the specific amplification of a piece of DNA from as few as 10 copies of a gene per gram of soil (Picard *et al*, 1996). PCR methods are substantially more sensitive, less expensive and less time-consuming than most alternative procedures (Lovell, 1994). Furthermore, PCR has been used extensively for the analysis of microbial populations in many environments, such as anaerobic reactors, marine sediments and roots (Clapp, 1999; Jeanthon *et al*, 1999a; Miguez *et al*, 1999).

McrA as marker gene for methanogens

The methanogen-specific gene used in this study was *mcrA*, which encodes a subunit of the enzyme methyl coenzyme M reductase. The use of *mcrA* as a marker gene for the methanogens has a number of advantages. Firstly, *mcrA* is believed to be unique to methanogens (Thauer, 1998), which means that primer or probes designed to encompass all *mcrA* sequences will detect only methanogens. Methanogen-specific 16S rDNA primers and probes can be designed (Raskin *et al*, 1994b). However, the probability of detecting sequences from non-methanogens or failing to detect some methanogen sequences is greater with 16S rDNA-based primers and probes.

Secondly, *mcrA* is more discriminating than 16S rDNA. Springer *et al* (1995) found that *mcrA* sequences of pairs of organisms had three times more changes than the respective pairs of 16S rRNA sequences. Lueders *et al* (2001) used *mcrA* to investigate methanogens in rice field soil, specifically because methods based on 16S rDNA had failed to resolve methanogenic and non-methanogenic lineages. Thirdly, *mcrA* is a functional gene, i.e. it encodes an enzyme involved in metabolism. This offers the possibility of measuring methanogenic activity by detecting *mcrA* mRNA. The level of expression of the enzymes involved in methanogenesis, such as methyl CoM reductase reflects methanogenic activity, and the level of transcription of the short-lived mRNA molecules is indicative of the level of enzyme expression (Hennigan & Reeve, 1994).

McrA has been used previously as a marker gene for methanogens (Hales *et al*, 1996; Hougaard and Westermann, 2000; Kudo *et al*, 1998; Lueders *et al*, 2001; Luton, 1996; Ohkuma *et al*, 1995; Springer *et al*, 1995). Lueders *et al*, (2001), Luton (1996) and Springer *et al* (1995) compared the phylogeny of *mcrA* with that of 16S rDNA and showed that the topology of phylogenetic trees based on the two genes were highly similar. In this study, we have added six additional *mcrA* sequences from described species, and a *mrtA* sequence, to the *mcrA/mrtA* gene database. Most notably, we have added three *mcrA* sequences from the family *Methanocorpusculaceae*, which was not represented in the *mcrA* database until now. The addition of these three sequences and the *mcrA* sequence from *Methanoculleus bourgensis* has doubled the number of *mcrA* sequences from the order *Methanomicrobiales*. Phylogenetic analysis of the new *mcrA* sequences (Figures 4.1 – 4.3, chapter 4) and comparison with the phylogeny of 16S rDNA sequences from members of the same genera

(Figure 1.8, chapter 1) showed similar topologies. Thus, providing further proof for the efficacy of *mcrA* as a phylogenetic marker for methanogens.

McrA and *mrtA*

Reeve *et al* (1997b) argued against the use of methyl reductase genes as phylogenetic markers for the methanogens because of the possible effects of two functionally equivalent genes. They stated that the presence of two methyl reductases raises the concern that in some species one of these enzymes may no longer be essential for methanogenesis or may have diverged sufficiently to catalyse a different reaction. Furthermore, having duplicate copies of a gene provides opportunities for gene conversion and therefore for unpredictable and possibly uneven sequence stabilisation (Reeve *et al*, 1997b). In addition, the possibility of lateral gene transfer, as appears to be the case for the *mrt* operon (Lehmacher and Klenk, 1994; Reeve *et al*, 1997b), could lead to the conclusion of incorrect phylogenetic relationships. Based on the phylogeny of *mrtA*, the *Methanobacteriales* appear to be closely related to the *Methanococcales*. Whereas, both *mcrA* and 16S rDNA indicate that these two orders are more distantly related (Figure 1.8, chapter 1 and Figures 4.1 – 4.3, chapter 4). Therefore, on its own *mrtA* would not be a good phylogenetic marker. However, in combination with *mcrA*, *mrtA* could provide useful additional information.

Bonacker *et al* (1992) showed that expression of the two methyl CoM reductases in *Methanothermobacter thermoautotrophicus* was strongly affected by growth conditions such as temperature, pH and substrate concentration. Nolling *et al* (1995), Pennings *et al* (1997) and Pihl *et al* (1994) showed that transcription of *mcr* and *mrt* genes was dependent on growth conditions. In particular, it was shown that the *mrt*

operon was transcribed preferentially under conditions of high H_2 availability, whereas the *mcr* operon was transcribed at a high level under H_2 limited conditions (Reeve *et al*, 1997a). Based on these findings, it may be possible to use the relative levels of *mcrA* and *mrtA* in environments such as landfill, to determine the growth conditions or metabolic activity of the methanogen population, or at least that part of the population that contained both *mcrA* and *mrtA*. One way in which *mcrA* and *mrtA* levels could be measured is with oligonucleotide probes, such as those designed in this study. However, the probes would need to target the same group or groups of methanogens.

To date, *mrtA* has been detected only in some species from the orders *Methanobacteriales* and *Methanococcales* (Reeve *et al*, 1997b; Springer *et al*, 1995). Temporal temperature gradient electrophoresis (TTGE) of PCR products amplified from 11 methanogens with the primers *mcrA*-P1 and *mcrA*-P3, appear to support this conclusion. PCR products from *Methanobacterium bryantii* and *Methanobacterium espanolae* were resolved into two bands by TTGE, while PCR products from species of *Methanobrevibacter, Methanocorpusculum, Methanoculleus, Methanohalophilus, Methanosaeta, Methanosarcina* and *Methanospirillum* ran as a single band. However, the possibility can not be ruled out that *mrtA* is present in these genera, but was not amplified or was amplified, but was not resolved by TTGE. Interestingly, *mrtA* accounted for 14% of the landfill clones, while *Methanobacteriales mcrA* accounted for just 4% of the clones. This discrepancy could be explained by amplification of *mrtA* from non-*Methanobacteriales*, or by preferential amplification of *mrtA* from *Methanobacteriales* over *mcrA* from *Methanobacteriales*. Lueders *et al* (2001) used

two primer pairs to amplify *mcrA/mrtA* genes. They noted that one primer pair failed to amplify *mrtA* from *Methanobacteriales* and *mcrA* from *Methanosaetaceae*.

Characterisation of landfill methanogen communities using molecular techniques

The molecular techniques employed in this study enabled the characterisation of the diversity and composition of the methanogenic community in the landfill environment. The PCR amplification of the *mcrA* gene enabled the specific detection of all methanogens without cultivation. Screening of clone libraries with PCR-RFLP provided a rapid measure of the diversity of the methanogen communities in each landfill sample, both in terms of the number of different OTUs and the relative abundance of each OTU. It was estimated that between 71% and 88% of the diversity present in the clone libraries was detected by the clones analysed (section 3.2.3.2, chapter 3). The phylogenetic analysis of *mcrA* sequences enabled the tentative identification of the members of the landfill methanogen communities, at least to the order level in the majority of cases and down to the species level in some cases. The results of the sequencing and phylogenetic analysis enabled the development of group-specific oligonucleotide probes for the *mcrA* and *mrtA* genes, which could be used for rapid characterisation of methanogen communities. Screening of the *mcrA/mrtA* clone libraries with these probes supported the results from the PCR-RFLP and phylogenetic analyses.

The results from all the analysis methods showed that the methanogen community diversity and structure was different for each of the landfill samples. Some samples such as the Hermitage leachate sample and the Brogborough 18m sample were dominated by one or two families, while other samples, notably the Poyle and

Brogborough 3m samples appeared to be much more diverse. Furthermore, where two samples were obtained from the same landfill, as was the case for the Brogborough and Hermitage landfills, the methanogen population in the two samples was substantially different, in terms of the relative abundance of the different families. This suggests that the methanogen community structure and diversity in each sample taken from a different location within a landfill, be it an excavated refuse sample or leachate sample, is likely to be unique to that sample. Furthermore, our results appear to indicate that there is not one species, genus or family of methanogens that are ubiquitous and abundant in landfill.

However, at the order level, *Methanomicrobiales* dominated in the majority of the samples. This order contains four families and nine genera of hydrogenotrophic methanogens (Table 1.5, chapter 1). The majority of species are also able to use formate and a number can utilise certain alcohols. Most species grow optimally at mesophilic temperatures, but psychrophilic and moderately thermophilic species have also been isolated. *Methanomicrobiales* have been detected in a wide variety of habitats, including landfills (Fielding and Archer, 1986; Fielding *et al*, 1988; Luton, 1996; Mori *et al*, 2000). The abundance of *Methanomicrobiales* in landfill has also been demonstrated using group-specific probes for methanogen 16S rDNA (M. van Dyke, personal communication). The orders *Methanobacteriales* and *Methanosarcinales* were detected at lower levels in the landfill samples, and in some cases they were not detected at all. *Methanbacteriales* is an order of hydrogenotrophic methanogens, some of which are able to utilise formate, and a few can use certain alcohols (Table 1.5, chapter 1). The order *Methanosarcinales* contains all the acetotrophic and methylotrophic species. Both *Methanobacteriales* and

Methanosarcinales have been detected previously in landfill (Fielding and Archer, 1986; Fielding *et al*, 1988; Luton, 1996). Members of the orders *Methanococcales* and *Methanopyrales* were not detected in any of the landfill samples. All the species of these two orders have so far been isolated only from marine or estuarine sources and would therefore not be expected to be present in terrestrial habitats.

Novel methanogens detected in landfill

In addition to those *mcrA/mrtA* sequences identified in landfill that showed close affiliation to described species, a number of groups of sequences were identified that may represent novel methanogens. Most notable among these unidentified groups was a small group of sequences that formed a cluster equidistant between the *Methanomicrobiales* and *Methanosarcinales* (Figures 4.1 – 4.3, chapter 4). The sequences in this cluster were closely related to *mcrA* sequences from uncultured *Archaea* detected in anaerobic digesters (Hougaard and Westermann, 2000). Unidentified clusters of sequences were also detected that grouped within the phylogenetic radiation of the *Methanobacteriales* and *Methanomicrobiales* (Figures 4.4 – 4.9, chapter 4). One group, the unidentified landfill *Methanomicrobiales mcrA* cluster 1, accounted for 24% and 21% of the clones in the Brogborough 3m and Poyle clone libraries respectively.

Genuine result or methodological bias ?

The dominance of hydrogenotrophic methanogens in landfill is interesting given that it has been calculated that the maximum theoretical contribution of H_2 to methanogenesis during anaerobic degradation of carbohydrate is 33% (Conrad, 1999). The remaining two-thirds of methane produced in nature originates from the methyl

group of acetate (Ferry, 1992). Possible explanations for H_2 contributing greater than 33% of methanogenesis include: 1) additional sinks of acetate; 2) additional sources of H_2; or 3) measurement under non-steady state conditions (Conrad, 1999). The first two explanations could both be satisfied if a reverse homoacetogenic reaction were taking place in landfills, i.e. the cleavage of acetate to form $H_2 + CO_2$. However, only a few homoacetogens have been observed to carry out such a reaction (Hattori *et al*, 2000; Schnurer *et al*, 1996), and its significance in nature is unknown. The last explanation could be true for the landfill samples from Odcombe and Brogborough. These samples were incubated in model landfill reactors prior to DNA extraction. It has been observed that, the low amounts of methane produced immediately after flooding of paddy soil are mainly due to H_2-dependent methanogenesis, since the hydrogenotrophic methanogens apparently become active before the acetotrophic ones (Conrad, 1999). The same scenario could apply to the start up of the model landfill reactors. If the hydrogenotrophic methanogen population in the reactors increased much more rapidly than the acetotrophic population after start up of the reactors, a bias would be observed towards hydrogenotrophic methanogens, such as the *Methanomicrobiales*. In addition, it has been shown for rice field soil samples that the relative contribution of $H_2 + CO_2$-dependent methanogenesis increased with increasing temperature and *vice versa* (Chin *et al*, 1999; Fey and Conrad, 2000). Therefore, incubation of the reactors at 37°C may also have led to an increase in the proportion of *Methanomicrobiales* relative to their *in situ* level. However, to counter this argument, it should be pointed out that *Methanosarcinales* were detected in both of the Brogborough samples, but they were not detected at all in the Hermitage leachate sample, from which DNA was extracted directly. *Methanosarcinales*

accounted for 10% of the Brogborough 3m sample, which is the second highest proportion of *Methanosarcinales* after the Hermitage excavated refuse sample.

In addition to the possible sources of bias outlined in the previous paragraph, other possible sources of bias should be borne in mind when interpreting the results. These include bias in the DNA extraction, due to preferential lysis of certain cell types, and bias in the PCR, due to preferential amplification of certain sequences. The PCR primers used in this study (*mcrA*-P1 and *mcrA*-P3) have been used to amplify *mcrA* from members of all five orders of methanogens, in this study, by P. Riley (personal communication) and by Luton (1996). This indicates that amplification with these primers should not exclude any methanogens, however, it does not rule out the possibility of preferential amplification of some groups. The harsh physical lysis method used in this study was chosen specifically because it was likely to work with all methanogen species. It was used successfully in this study and by P. Riley (personal communication) to extract DNA from a range of methanogen pure cultures. The same method has been used by Daly *et al* (2000) to extract DNA from landfill leachate samples, for the investigation of SRB.

It would be of interest to determine if any of the possible sources of bias may have affected the results. PCR bias could be investigated by comparing results obtained with different primer pairs, such as those described by Hales *et al* (1996) and Springer *et al* (1995). Similarly, bias in the DNA extraction could be investigated by comparing the results from different DNA extraction methods. It would be particularly interesting to perform a comparison of direct DNA extraction versus DNA extraction after incubation of the sample in a model landfill reactor, at different

temperatures, and for different incubation periods. Although both methods were used in this study, they were not applied to the same sample.

Acetogens in landfill

The issue of homoacetogens in landfill was addressed by the detection of the FTHFS gene in landfill samples. However, further work is required to determine if homoacetogens were detected by the PCR and probing, or FTHFS-producing non-acetogens. The PCR primers for the FTHFS gene described by Leaphart and Lovell (2001) could be used to investigate homoacetogens in landfill, using the same techniques employed for the methanogens.

A second group of acetogenic *Bacteria*, the syntrophic-acetogens, is believed to be active in the landfill degradation process. Like the homoacetogens, the syntrophic-acetogens have received little attention in landfill. It would be of interest to investigate this group in landfill, since they may be responsible for a large part of the acetate, hydrogen and carbon dioxide production in landfills. The seven described genera of syntrophic-acetogens are from two taxonomic groups, the delta sub-class of the Proteobacteria and the low G+C sub-class of Gram positive bacteria. 16S rRNA-based oligonucleotide probes have been described in the literature and used to detect syntrophic-acetogens in anaerobic reactors (Hansen *et al*, 1999; Harmsen *et al*, 1996).

Conclusions

Through the development and use of molecular methods this study has greatly increased our knowledge of the diversity and structure of methanogen communities in landfill. Phylogenetic analysis of *mcrA* sequences from landfill samples identified the

presence of methanogens related to previously described species, in addition to several possibly novel methanogen groups. The order *Methanomicrobiales* appeared to be the most abundant and diverse group in landfill. The PCR-RFLP technique proved to be a simple and efficient method for screening clone libraries, and determining the diversity and structure of methanogen communities. In addition, *mcrA* PCR products digested with *Taq*I could be used in other techniques such as T-RFLP. A set of group-specific oligonucleotide probes for the *mcrA* and *mrtA* genes were designed and successfully applied to the screening of *mcrA/mrtA* clone libraries. These probes enabled rapid description of the methanogen communities in landfill samples, and should prove to be a versatile and valuable tool for the quantitation and characterisation of methanogen communities in landfill and other environments.

Future work

The results of this study have raised a number of questions regarding the methanogen community in landfills. Firstly, given the heterogeneity of the methanogen community between landfills and within landfills, is it possible to establish a link between the composition of a methanogen community and the degradation process in a landfill? Secondly, is the apparent predominance of hydrogenotrophic *Methanomicrobiales* species common in landfills, and what does this tell us about the microbial process occurring in landfill? The molecular techniques developed in this study could prove useful for answering these and other questions.

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APPENDIX A.

Alignment of nucleotide sequences determined in this study for *mcrA* PCR products from landfill samples.

Landfill samples from which *mcrA* sequences were amplified:

MS Excavated refuse sample from Mucking site.

OS Excavated refuse sample from Odcombe site.

BSD Excavated refuse sample from Brogborough site, depth 18m.

BSS Excavated refuse sample from Brogborough site, depth 3m.

PL Leachate sample from Poyle site.

HL Leachate sample from Hermitage site.

The alignment was created using PILEUP (GCG Wisconsin Package 10.1, Genetics Computer Group, Wisconsin, USA).

Gap creation penalty: 1

Gap extension penalty: 1

```
        1                                                   50
MS19    TACACTGATA ACATCCTCGA CGACTTTACT TACTATGGTA AAGAATACGT
MS26    TACACTGATA ACATCCTCGA CGACTTTACT TACTATGGTA AAGAATACGT
OS111   TACACTGATA ACATCCTCGA CGACTTTACC TACTATGGTA AAGAATACGT
OS61    TACACTGATA ACATCCTCGA CGACTTTACC TACTATGGTA AAGAATACGT
MS6     TACACTGATA ACATCCTCGA CGACTTTACC TACTATGGTA AAGAATACGT
BSS2    TACACTGATA ACATCCTCGA CGACTTTACC TACTATGGTA AAGAATACGT
BSS49   TACACTGACA ACGTACTCGA CGACTTCACC TACTTTGGTA AAGAGTACGT
BSS54   TACACTGACA ACGTACTCGA CGACTTCACC TACTTTGGTA AAGAGTACGT
OS105   TACACTGACA ATATCCTTGA CGACTTCACC TACTTCGGTA AAGAGTACGT
OS108   TACACTGACA ATATCCTTGA CGACTTCACC TACTTCGGTA AAGAGTACGT
OS82    TACACCGACA ACATCTTAGA CGACTTCACT TACTACGGTA AAGAATACGT
BSS22   TACACTGACG ATATCCTGGA TGACTTCGTA TACTACGGAA TGGAATACGT
BSS26   TACACTGACG ATATCCTGGA TGACTTCGTA TACTACGGAA TGGAATACGT
MS28    TACACTGACG ATATCCTGGA TGACTTCGTA TACTACGGAA TGGAATACGT
OS102   TACACTGACG ATATCCTGGA TGACTTCGTA TACTACGGAA TGGAATACGT
OS25    TACACTGACG ATATCCTGGA TGACTTCGTA TACTACGGAA TGGAATACGT
MS51    TACACTGACG ATATCCTGGA TGACTTCGTA TACTACGGAA TGGAATACGT
OS37    TACACTGACG ATATCCTGGA TGACTTCGTA TACTACGGAA TGGAATACGT
OS70    TACACCGACG ATATACTGGA CGACTTCCTC TACTACGGAA TGGAATACGT
BSS12   TACACAGACG ACATACTTGA CGACTTCCTT TACTACGGTA AAGAATACAT
BSS50   TACACTCATC ATATCCTCCA CAACAACCTC TACTACCACC TTCACTACAT
BSS65   TACACTGATG ATATCCTCGA CAACAACGTG TACTACGACG TTGACTACAT
MS22    TACACCGATG ACATCCTCGA CAACAACGTG TACTACGACG TTGACTACAT
MS42    TACACAGATG ACATCCTCGA CAACAACGTG TACTACGACG TTGACTACAT
BSS21   TACACTGACG ACATCCTTGA CAACAACGTG TACTACAACG TTGACTACAT
BSS8    TATACTGACA ACATCCTCGA TGATTTCACG TACTACGGGA TGGACTATCT
BSS9    TATACTGATA ACATCCTCGA TGATTTCACG TACTACGGGA TGGACTATCT
MS16    TACACCGACA ACATCCTCGA TGAGTTCACC TACTACGGTA TGGACTACAT
MS37    TACACCGACA ACATCCTCGA TGAGTTCACC TACTACGGTA TGGACTACAT
MS23    TACACCGACA ACATCCTCGA TGAGTTCACC TACTATGGTA TGGACTACAT
OS48    TACACCGACA ACATCCTCGA TGAGTTCACC TACTACGGTA TGGACTACAT
OS27    TACACGGATA ACATCCTTGA TGACTTTATC TACAGTGGAA TGGACTACAT
OS71    TACACGGATA ACATCCTTGA TGACTTCACT TACTATGGAA TGGACTACCT
OS58    TACACGGATA ACATCCTTGA TGACTTTATC TACAGCGGAA TGGACTACAT
OS65    TACACGGATA ACGTCCTTGA TGACTTTATC TACAGCGGAA TGGACTACAT
PL3     TACACGGATA ACGTCCTTGA TGACTTTATC TACAGCGGAA TGGACTACAT
HL110   TACACGGATA ACATCCTTGA TGACTTCACC TACTACGGAA TGGACTACCT
OS41    TACACGGATA ACATCCTTGA TGACTTCACC TACTACGGAA TGGACTACCT
OS110   TACACGGATA ACGTCCTTGA TGACTTCACC TACTACGGAA TGGATTACCT
PL126   TACACGGATA ACATCCTTGA TGACTTTGTA TACAGCGGTA TGGACTACAT
OS15    TACACCGATA ACATCCTTGA TGACTTCATC TACCATGGAA TGGACTACCT
OS20    TACACCGATA ACATCCTTGA TGACTTCATC TACCATGGAA TGGACTACCT
OS59    TACACCGATA ACATCCTCGA TGACTTCATC TACCATGGAA TGGACTACCT
OS80    TACACCGATA ACATCCTCGA TGACTTCATC TACCACGGTA TGGACTACCT
HL99    TACACCGATA ACATTCTCGA TGACTTCACG TATCATGGAA TGGACTACCT
OS63    TACACGGATA ACATCCTTGA TGACTTTACC TACTACGGTA TGGATTACAT
HL34    TACACGGATA ACATCCTTGA TGACTTCACT TACTATGGAA TGGATTACCT
BSD28   TACACAGACA ACATCCTCGA CGAGTTCACA TACTACGGTA TGGACTACAT
PL109   TACACCGACA ACATCCTTGA TGACTTCACC TACTACGGTA TGGACTACAT
PL187   TACACCGACA ACATCCTTGA TGACTTCACC TACTACGGTA TGGACTACAT
PL21    TACACCGACA ACATCCTTGA TGACTTGACC TACTACGGTA TGGACTACAT
PL206   TACACCGACA ACATCCTCGA TGAGTTCACC TACTACGGTA TGGACTACAT
PL40    TACACCGACA ACATCCTTGA TGAGTTCACC TACTACGGTA TGGACTACAT
BSS14   TACACCGATA ACATCCTCGA TGACTTTACC TACTACGGTA TGGATTACAT
BSS43   TACACCGACA ACATCCTTGA TGACTTTACC TACTACGGTA TGGACTACAT
BSD29   TACACCGACA ACATCCTCGA TGAGTTCACC TACTACGGTA TGGACTACAT
BSD63   TACACCGACA ACATCCTCGA TGAGTTCACC TACTACGGTA TGGACTACAT
BSD95   TATACCGACA ACATCCTTGA TGAGTTCACC TACTACGGTA TGGACTACAT
BSS46   TACACAGACA ACATCCTCGA CGAGTTCACC TACTACGGTA TGGACTACGT
BSD21   TACACCGACA ACATCCTCGA TGAGTTCACC TACTACGGTA TGGACTACAT
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BSD42   TACACCGACA ACATCCTCGA TGAGTTCACC TACTACGGTA TGGACTACAT
BSD14   TACACCGACA ACATCCTCGA TGAGTTCACC TACTACGGTA TGGACTACAT
BSD67   TACACCGACA ACATCCTCGA CGAGTTCACC TACTACGGTA TGGACTACAT
BSD73   TACACCGATA ACATCCTCGA CGAGTTCACC TACTACGGTA TGGACTACAT
BSD90   TACACCGACA ACATCCTCGA CGAGTTCACC TACTACGGTA TGGACTACGT
HL81    TACACCGACA ACATCCTCGA CGAGTTCACC TACTACGGTA TGGACTACGC
PL240   TACACCGACA ACATCCTCGA CGAGTTCACC TACTACGGTA TGGACTACGC
PL22    TACACCGACA ACATCCTGGA CGAGTTCACC TACTACGGGA TGGACTACGC
BSD61   TACACCGACA ACATCCTCGA CGAGTTCACC TACTACGGTA TGGACTACGT
MS61    TACACCGACA ACATCCTCGA CGAGTTCACC TACTACGGTA TGGACTACGC
BSS52   TACACCGACA ACATCCTTGA CGACTTCACC AGCTACGGTG TCGACTACGT
BSS59   TACACCGACA ACATCCTTGA CGACTTCACC AGCTACGGTG TCGACTACGT
BSS74   TACACCGACA ACATCCTTGA CGACTTCACC AGCTACGGTG TCGACTACGT
PL145   TACACCGACA ACATCCTTGA CGACTACTGC TACTATGGTC TTGACTACGT
PL181   TACACCGACA ACATCCTTGA CGACTACTGC TACTATGGTC TTGACTACGT
HL108   TACACCGACA ACATCCTCGA TGACTACTGC TACTATGGTC TTGACTACAT
PL234   TACACCGACA ACATCCTCGA TGACTACTGT TACTATGGTC TTGACTACAT
PL1     TACACGGACA ACATCCTCGA TGACTACTGC TACTATGGTC TTGATTACAT
PL36    TACACCGACA ACATCCTCGA TGACTACTGT TACTATGGTC TTGACTACAT
PL238   TACACCGACA ACATCCTTGA CGACTACTGC TACTATGGTC TTGACTACAT
PL7     TACACGGACA ACATCCTTGA CGACTACTGC TACTACGGTC TTGACTATGT
PL43    TACACCCACA ACATCCTCCA TCACTACTGC TACTATGGTC TTGACTATGT
PL225   TACACCGACA ACATCCTTGA CGACTTCTGC TACTATGGTC TTGACTACAT
OS77    TACACCGACA ACATCCTCGA CGAGTTCACC TACTACGGTA TGGACTACTT
OS18    TACACCGACA ACATCCTGGA GGACTACGTC TACTACGCCA TCGACACCAT
OS55    TACACCGACA ACATCTTGGA GGACTACGTC TACTACGCCA TCGACACCAT
HL74    TACACGGATA ACATCCTTGA GGACTACACC TATTACGCGA TCGACTACAT
BSD43   TACACCAACG ATGTCCTGGA CGACTTCTGC TACTACGGCG TCGATTTCGC
BSD79   TACACCAACG ATGTCCTGGA CGACTTCTGC TACTACGGCG TCGATTTCGC
PL53    TACACCAACG ATGTCCTCGA CGACTTCTCC TACTATGCAG CTGACTACGC

        51                                                  100
MS19    TGAAGATAAA TAC..GGTGG GCTCACGGAA ..........  .........G
MS26    TGAAGATAAA TAC..GGTGG GCTCACGGAA ..........  .........G
OS111   CGAAGATAAA TAC..GGTGG ACTCACGGAA ..........  .........G
OS61    CGAAGATAAA TAC..GGTGG ACTCACGGAA ..........  .........G
MS6     CGAAGATAAA TAC..GGTGG ACTCACGGAA ..........  .........G
BSS2    CGAAGATAAA TAC..GGTGG ACTCACGGAA ..........  .........G
BSS49   AGAAGATAAA TAC.....GG AATGACCGAA ..........  .........G
BSS54   AGAAGACAAA TAC.....GG AATGACCGAA ..........  .........G
OS105   GGAAGACAAA TAC.....GG AATCACTGAA ..........  .........G
OS108   GGAAGACAAA TAC.....GG AATAACTGAA ..........  .........G
OS82    CGAAGATAAA TTC.....GG AATGACTGAA ..........  .........G
BSS22   GGACGACAAA TAC..GGT.. .ATCTGTGGA ..........  .........A
BSS26   GGACGACAAA TAC..GGT.. .ATCTGTGGA ..........  .........A
MS28    GGACGACAAA TAC..GGT.. .ATCTGTGGA ..........  .........A
OS102   GGACGACACA TAC..GGT.. .ATCTGTGGA ..........  .........A
OS25    GGACGACAAA TAC..GGT.. .ATCTGTGGA ..........  .........A
MS51    GGACGACAAA TAC..GGT.. .ATCTGTGGA ..........  .........A
OS37    GGACGACAAA TAC..GGT.. .ATCTGTGGA ..........  .........A
OS70    GGACGGTAAA TAC..GGT.. .ATCTGTGGA ..........  .........A
BSS12   CGAAGACAAG TAC..GGA.. .ATGTGCGGA ..........  .........G
BSS50   CAACGACAAG TACAACGGTG .CTGCAA..A CCTCGGAACT GACAACAAGG
BSS65   CAACGACAAG TACAACGGTG .CTGCAA..A CCTCGGAACT GACAACAAGG
MS22    CAACGACAAG TACAACGGTG .CTGCAA..A CCTCGGCAAG GACAACAAAG
MS42    CAACGACAAG TACAACGGTG .CTGCAA..C CGTCGGCAAG GACAACAAGA
BSS21   CAACGACAAG TACAATGGAG .CTGCAA..A CATAGGCACT GACAACAAGG
BSS8    CAAGGACAAG TACGGCTACA ATTACCGCGA ACCGGGCCCG GACAGGGTTA
BSS9    CAAGGACAAG TACGGCTACA ATTACCGCGA ACCGGGCCCG GACAGGGTTA
MS16    CAAGGACAAG TACAAAGTCG ACTGGAAGGC ATGTAACCCA GCAGACAAGG
MS37    CAAGGACAAG TACAAAGTCG ACTGGAAGGC ATGTAACCCA GCAGACAAGG
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MS23    CAAGGACAAG TACAAAGTCG ACTGGAAGGC ATGTAACCCA GCAGACAAGG
OS48    CAAGGACAAG TACAAAGTCG ACTGGAAGGC ATGTAACCCA GCAGACAAGG
OS27    CCACGACAAG CACAAGATCG ACACCAAGAA TCCGAACCCG AACGACAAAG
OS71    CCACGACAAG TACAAGATCG ACACCAAGAA CCCGAACCCG AACGACAAAG
OS58    CCACGACAAG TACAAGATCG ACTGGAAGAA CCCGAACCCG AAAGACAAAG
OS65    CCACGACAAG TACAAGATCG ACTGGAAGAA CCCGAACCCG AAAGACAAAG
PL3     CCACGACAAG TACAAGATCG ACTGGAAGAA CCCGAACCCG AAAGACAAAG
HL110   CCACGACAAG TACAAGGTCG ACACCAAGAA TCCGAACGCA AAAGACAAAG
OS41    CCACGACAAG TACAAGGTCG ACACCAAGAA TCCGAACGCA AAAGACAAAG
OS110   CCACGACAAG TACAAGATTG ACTGGAAGAA CCCGAACCCG AAAGACAAAG
PL126   CCACGACAAG TACAACGTCG ACCTCAAGAA CCCGAACCCG AACGACAAAG
OS15    CCACGACAAA TACAAGATCG ACTGGAAGAA CCCAAGCCCG GCAAACAATG
OS20    CCACGACAAA TACAAGATCG ACTGGAAGAA CCCAAGCCCG GCAAACAATG
OS59    CCACGACAAA TACAAGATCG ACTGGAAGAA CCCAAGCCCG GCAAACAATG
OS80    CCACGACAAA TACAAGATCG ACTGGAAGAA CCCAAGCCCG GCAAACAATG
HL99    CCACGACAAA TACAAGATCG ACTGGAAGAA CCCGAACCCG AAAGACAAGG
OS63    CCACGACAAG TACAAGGTCG ACCTCAAGAA CCCGAACCCG AACGACAAAG
HL34    CCATGACAAG TACAAGATCG ATGTTAAGAA CCCGAACCCG AAAGACAAGG
BSD28   CAAAGACAAA TACAGCGTCG ACTACACACA CCCAAGCCCC ACAGACACTG
PL109   CAAGAGCAAG TACAAAGTCG ACTGGAAGAA CCCGTCCGGC AAGGACCGTG
PL187   CAAGAGCAAG TACAAGGTTG ACTGGAAGAA CCCGTCCGAC AAGGACCGTG
PL21    CAACAACAAC TACAAACTCC ACTCCAACAA CCCCTCCCAC AACCACCCTC
PL206   CAAGGACAAG TACAAGGTCA ACTGGAAGAG CCCGTCCGAC AAGGACAAGC
PL40    CAAGGACAAA TACAAGGTCA ACTGGAAGAG CCCATCCGAC AAGGACAAGC
BSS14   CAAGGACAAG TACAAAGTCG ACTGGAAGGC CCCGTCAGAC AAGGACAAGG
BSS43   CAAGAACAAG TACAAAGTCG ACTGGAAGAG CCCGTCCGGC AAGGACAAGG
BSD29   CAAGGACAAG TACAAGGTCG ACTTCAAGAA CCCAAGCGCT AAGGACAAGG
BSD63   CAAGGACAAG TACAAGGTCG ACTTCAAGAA CCCAAGCGCT AAGGACAAGG
BSD95   CAAGGACAAG TACAAGGTCG ACTGGAAGAA CCCGAGCGCG AAGGACAAGA
BSS46   CAAGGACAAG TACACAGTCG ACTGGAAGAA CCCGAGCCCG AAGGACAAGG
BSD21   CAAGGACAAG TACAAGGTCG ACTGGAAGAA CCCAAGCGCG AAGGACAAGA
BSD42   CAAGGACAAG TACAAGGTCG ACTGGAAGAA CCCGAACGCG AACGACAAGG
BSD14   CAAGGACAAG TACAAGGTCG ACTGGAAGAA CCCGAACGCG AACGACAAGG
BSD67   CAAGGACAAG TACAAGGTCG ACTGGAAGAA CCCGAGCCCG AAGGACAAGG
BSD73   CAAGGACAAG TACAAGGTCG ACTGGAAGAA CCCGAGCCCG AAGGACAAGG
BSD90   CAAGGACAAG TACAAGGTCG ACTGGAAGAA CCCGAGCGCG AAGGACAAGG
HL81    CAAGGACAAG TACAAGGTCG ACTGGAAGAA CCCGAGCCCG AGCGACAAGG
PL240   CAAGGACAAG TACGGGGTCG ACTGGAGAAA CCCGAGCCCC AAGGACAAGG
PL22    CAAGGACAAG TTTGGGGTCG ACTGGAAGAA CCCGAGCCCG AACGACAAGG
BSD61   CAAGGACAAG TACAAAGTCG ACTGGAAGAA CCCGAGCCCG AAGGACAAGG
MS61    CAAGGACAAG TACAAGGTCG ACTGGAAGAA CCCCAGCCCG AGCGACAAGG
BSS52   CAAGAAGAAG CAC..GGCGC TCT..... .......... ..CGGCAAGG
BSS59   CAAGAAGAAG CAC..GGCGC TCT..... .......... ..CGGCAAGG
BSS74   CAAGAAGAAG CAC..GGCGC TCT..... .......... ..CGGCAAGG
PL145   CAAGAAGAAC CAT..GGTGG TCT..... .......... ..CGGCAAGG
PL181   CAAGAAGAAC CAT..GGTGG TCT..... .......... ..CGGCAAGG
HL108   CAAGTCCAAG CAC..GGCGG TCT..... .......... ..CGGAAAGG
PL234   CAACTCCAAG CAC..GGTGG CAT..... .......... ..CGGCAAGG
PL1     AAACTCCAAG CAC..GGCGG TCT..... .......... ..CGGCAAGG
PL36    CAAGTCCAAG CAC..GGCGG CAT..... .......... ..CGGCAAGG
PL238   CAACAAGAAC CAC..GGTGG TCT..... .......... ..CGGCAAGG
PL7     AAAGAAGAAC CAC..GGTGG CAT..... .......... ..CGGCAAGG
PL43    AAAGAAGAAC CAC..GGTGG TCT..... .......... ..CGGCAAGG
PL225   CAAGTCCAAG CAC..GGGGG CAT..... .......... ..CGGCAAGG
OS77    AAAGGACAAG TAC..GGCGG CTA..... .......... ..CTCGCAGG
OS18    AAAGGACAAG TCTGGCGGCT TCTGCAAG.. .CTTGACCCG AACAAC....
OS55    AAAGGACAAG TATGGCGGCT TCTGCAAG.. .CTTGACCCG AACAAC....
HL74    CAAAGACAAG TACGGCGGCT TCTGCAAG.. .CTTGACCCG AACAAC....
BSD43   CAACGACAAG TTCGGCGGAT TCGCCAAG.. ...GCACCCA AGCTGC....
BSD79   CAACGACAAG TTCGGCGGAT TCGCCAAG.. ...GCACCCA AGCTGC....
PL53    AGTAGACAAG TTCGGCGGAT TTGCAAAG.. ...GCACCCG CAACTG....
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        101                                                        150
MS19    CACCAAACAA CATGGACACT ATTCTGGACG TAGCATCAGA AGTCACATTC
MS26    CACCAAACAA CATGGACACT ATTCTGGACG TAGCATCAGA AGTCACATTC
OS111   CACCAAACAA CATGGACACT ATTCTGGACG TAGCATCAGA AGTCACATTC
OS61    CACCAAACAA CATGGACACT ATTCTGGACG TAGCATCAGA AGTCACATTC
MS6     CACCAAACAA CATGGACACT ATTCTGGACG TAGCATCAGA AGTCACATTC
BSS2    CACCAAACAA CATGGACACT ATTCTGGACG TAGCATCAGA AGTCACATTC
BSS49   CACCAAACAC CATGGACACT GTCCTGGACG TTGCTTCCGA AGTTAACTTC
BSS54   CACCAAACAC CATGGACACT GTCCTGGACG TTGCTTCCGA AGTTAACTTC
OS105   CACCAAACAA CATGGACACT GTCCTGGATG TTGCATCAGA AGTAACTTTC
OS108   CACCAAACAA CATGGACACT GTCCTGGATG TTGCATCAGA AGTAACCTTC
OS82    CACCTAACAA TATGGACACA GTCCTAGATG TAGGATCAGA AGTTACATTC
BSS22   CCAAAGCAAC CACTGAAGTG GTTCACGACA TAGCCTCAGA AGTAACCATG
BSS26   CCAAAGCAAC CACTGAAGTG GTTCACGACA TAGCCTCAGA AGTAACCATG
MS28    CCAAAGCAAC CACTGAAGTG GTTCACGACA TAGCCTCAGA AGTAACCATG
OS102   CCAAAGCAAC CACTGAAGTG GTTCACGACA TAGCCTCAGA AGTAACCATG
OS25    CCAAAGCAAC CACTGAAGTG GTTCACGACA TAGCCTCAGA AGTAACCATG
MS51    CCAAAGCAAC CACTGAAGTG GTTCACGACA TAGCCTCAGA AGTAACCATG
OS37    CCAAAGCAAC CACTGAAGTG GTTCACGACA TAGCCGCAGA AGTAACCATG
OS70    CCAAAGCAAC CACTGAAGTG GTTCACGACA TAGCCGCAGA AGTAACCATG
BSS12   CAAAACCAAG CATGGACGTT GTCAAAGACA TAGCAAGTGA AGTTACACTC
BSS50   TAAAGGCAAC CCTCGATGTA GTAAAGGACA TCGCAACCGA GTCCACACTC
BSS65   TAAAGGCAAC CCTCGATGTA GTAAAGGACA TCGCAACCGA GTCCACACTC
MS22    TAAAGGCAAC CCTCGACGTC GTAAAGGACA TCGCAACCGA GTCCACAATC
MS42    TAAAGGCAAC TCTGGAAGTC GTAAAGGACA TCGCAACCGA ATCCACAATC
BSS21   TAAAGGCAAC TCTCGATGTC GTAAAGGATA TCGCAACCGA GTCCACACTC
BSS8    TTAAGCCCAC GCAGGAGATC GTAAACGACC TGGCAACGGA GGTTTGTCTC
BSS9    TTAAGCCCAC GCAGGAGATC GTAAACGACC TGGCAACGGA GGTTTGTCTC
MS16    TCAAACCAAC TCAGGAAGTC GTCAACGACA TTGCCGGAGA GGTCACCCTC
MS37    TCAAACCAAC TCAGGAAGTC GTCAACGACA TTGCCGGAGA GGTCACCCTC
MS23    TCAAACCAAC TCAGGAAGTC GTCAACGACA TTGCCGGAGA GGTCACCCTC
OS48    TCAAACCAAC TCAGGAAGTC GTCAACGACA TTGCCGGAGA GGTCACCCTC
OS27    TCAAAGCAAC TCAGGAAGTT GTCAACGACA TTGCAAGCGA AGTCAACCTT
OS71    TCAAAGCAAC TCAGGAAGTT GTCAACGACA TTGCAAGCGA AGTCAACCTT
OS58    TCAAAGCAAC GCAGGAAGTT GTCAACGACA TTGCAACCGA AGTCAACCTT
OS65    TCAAAGCAAC CCAGGAAGTT GTCAACGACA TTGCAACCGA AGTCAACCTT
PL3     TCAAAGCAAC CCAGGAAGTT GTCAACGATA TTGCAACCGA AGTCAACCTT
HL110   TCAAAGCAAC CCAGGAAGTT GTCAACGACA TCGCAACCGA AGTCAACCTT
OS41    TCAAAGCAAC CCAGGAAGTT GTCAACGACA TCGCAACCGA AGTCAACCTT
OS110   TCAAAGCAAC CCAGGAAGTT GTCAACGACA TCGCAACCGA AGTCAACCTT
PL126   TCAAAGCAAC CCAGGAAGTT GTCAACGACA TCGCAACCGA AGTCAACCTT
OS15    TTGCAGCAAC TCAGGAAGTT GTCAACGACA TTGGTACTGA AGTCAACCTT
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OS59    TTGCAGCAAC TCAGGAAGTT GTCAACGACA TTGGTACTGA AGTCAACCTT
OS80    TTGCAGCAAC CCAGGAAGTT GTCAACGACA TTGGTACTGA AGTCAACCTT
HL99    TCAAAGCAAC CCAGGAAATC GTCAACGACA TTGCGACCGA AGTCAACCTT
OS63    TCAAAGCAAC CCAGGAAGTT GTCAACGACA TTGCAACCGA AGTCAACCTT
HL34    TCAAAGCAAC TCAGGAAGTT GTCAGCGACA TTGCAACTGA AGTCAACCTT
BSD28   TCAGGCCAAC ACAGGATGTT GTCAACGACA TCGCAACAGA GGTCAACCTA
PL109   TCAAGCCGAC CCAGGACATT ATCAACGAGC TCGCAACCGA GGTCACCCTC
PL187   TCAAGCCCAC CCAGGACCTT ATCAACGAGC TCGCAACCGA GGTCACCCTC
PL21    TCAAGCCGAC CCAGGATCTT GTCAACGAGC TCGCAACCGA GGTCACCCTC
PL206   TCAAGCCGAC CCAGGACCTG GTAAACGAGC TTGCCTCGGA AGTCACCCTC
PL40    TCAAGCCGAC CCAGGACCTG GTAAACGAGC TCGCCTGGGA AGTCACCCTC
BSS14   TCAAGCCAAC CCAGGAACTG GTAAACGAGC TTGCATCCGA AGTTACCCTC
BSS43   TCAAGCCGAC CCAGGAACTG GTAAACGAGC TCGCCTCGGA AGTCACCCTC
BSD29   TCAAGGCAAC CCAGGACGTT GTTAATGACA TTGCAACCGA GGTCACCCTC
BSD63   TCAAGGCAAC CCAGGACGTT GTTAATGACA TTGCAACCGA GGTCACCCTC
BSD95   TCAAGCCGAC CCAGGATGTC GTCAACGACA TGGCAACCGA GGTCACCCTC
BSS46   TCAAGCCGAC CCAGGAGATC GTCAACGACA TGGCGACCGA GGTTACCCTC
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BSD21   TCAAGCCGAC CCAGGATGTC GTCAACGACA TGGCAACCGA GGTCACCCTC
BSD42   TCAAGCCGAC CTACGACGTC GTCAACGACA TGGCAACCGA GGTCACCCTC
BSD14   TCAAGCCGAC CTACGACGTC GTCAACGACA TGGCAACCGA GGTCACCCTC
BSD67   TCAAGCCGAC CTACGATATC GTCAACGACA TCGCAACCGA GGTTGCCCTC
BSD73   TCAAGCCGAC CTACGATATC GTCAACGACA TCGCAACCGA GGTTGCCCTC
BSD90   TCAAGCCGAC CTACGACGTC GTCAACGACA TGGCAACCGA GGTTGCCCTC
HL81    TCAAGCCGAC CCAGGGGATC GTCAACGATC TCGCAACCGA GGTCACCCTC
PL240   TCAAGCCGAC CCAGGAGATC GTCAACGATC TCGCAACCGA GGTCACCCTC
PL22    TCAAGCCGAC CCAGGAGATC GTCAACGACC TGGCCACCGA GGTCACCCTC
BSD61   TCAAGCCGAC CCAGGAGATC GTCAACGACA TGGCGACCGA GGTCACCCTC
MS61    TCAAGCCCAC CCAGGAGATC GTCAACGACC TCGCGACCGA AGTCACCCTC
BSS52   TCAAGGCAAC CCAGGACGTT GTCAACGACA TCGCTTCCGA GGTCACCCTC
BSS59   TCAAGGCAAC CCAGGACGTT GTCAACGACA TCGCTTCCGA GGTCACCCTC
BSS74   TCAAGGCAAC CCAGGACGTT GTCAACGACA TCGCTTCCGA GGTCACCCTC
PL145   CAAAGGCGAC CCAGGAAGCA GTCACTGACA TTGCATCCGA AGTCACCCTC
PL181   CAAAGGCGAC CCAGGAAGCA GTCACTGACA TTGCATCCGA AGTCACCCTC
HL108   CAAAGAAGAC CCAGGAAGTT ATCAGCGACA TCGCAACCGA AGTCACCCTC
PL234   CAAAGAAGAC CCAGGAAGTT GTCAACGACA TCGCAACCGA GGTCACGCTC
PL1     CAAAAAAGAC TCAGGAAGTC GTCAACGACA TCGCAACCGA GGTCACGCTC
PL36    CAAAGCAGAC CCAGGAAGTC GTCAACGACA TCGCAACCGA AGTTACGCTC
PL238   CAAAGCACAC CCAGGAAGTT ATCAACGACA TCGCAACCGA AGTCACCCTC
PL7     CAAAGGCGAC CCACGATCCA CTCAACCACA TCGCAACCCA GCTTACCCTT
PL43    CAAAGAAGAC CCAGGAAGCA ATCAACGACA TCGCAACCGA AGTCACCCTC
PL225   CAAAGGCAAC CCAGGAAACC GTCAACGACA TCGCTACCGA GGTTACCCTC
OS77    CACCAGCAAC CCAGGAGGTC GTCAACGACC TCGCAACTGA GGTCACGATG
OS18    .......... .TACGACAAG CTGATGGAGC TCGGAGACAA CGTCAACACC
OS55    .......... .TATGACAAG CTGATGGAGC TCGGAGACAA CGTCAACACC
HL74    .......... .TACGATGAG ATGATGAAGC TCGGTTACAA CGTCAACTCA
BSD43   .......... .T.GGATACC GCCAAGGAGC TGGCCACTGA GGTCAACGCC
BSD79   .......... .T.GGATACC GCCAAGGAGC TGGCCACTGA GGTCAACGCC
PL53    .......... .T.CGAGACT GCCAAGGACA TCGCCACTGA GGCTACCCTC

        151                                                 200
MS19    TACGGTCTAG AACAGTACGA AGAATTCCCA GCACTCTTAG AAGATCA.AT
MS26    TACGGTCTAG AACAGTACGA AGAATTCCCA GCACTCTTAG AAGATCA.AT
OS111   TACGGTCTTG AACAGTACGA AGAATTCCCA GCACTCTTAG AAGATCA.GT
OS61    TACGGTCTTG AACAGTACGA AGAATTCCCA GCACTCTTAG AAGATCA.GT
MS6     TACGGTCTTG AACAGTACGA AGAATTCCCA GCACTCTTAG AAGATCA.GT
BSS2    TACGGTCTAG AACAGTACGA AGAATTCCCA GCACTCTTAG AAGATCA.AT
BSS49   TACGCACTGG AACAGTTCGA AGACTACCCA GCATTACTCG AAA.CCATAT
BSS54   TACGCACTGG AACAGTTCGA AGACTACCCA GCATTACTCG AAA.CCATAT
OS105   TACGCACTCG AACAGTTCGA AGACTACCCC GCACTCCTCG AAA.CCATAT
OS108   TACGCACTTG AACAGTTCGA AGACTACCCC GCACTCCTCG AAA.CCATAT
OS82    TATGCTCTAG AACAGTTCGA AGAATACCCA GCACTCTTAG AAA.CCATCT
BSS22   TACGGACTGG AACAGTACGA ...ATACCCA GCACTCATGG AAGACCA.CT
BSS26   TACGGACTGG AACAGTACGA ...ATACCCA GCACTCATGG AAGACCA.CT
MS28    TACGGACTGG AACAGTACGA ...ATACCCA GCACTCATGG AAGACCA.CT
OS102   TACGGACTGG AACAGTACGA ...ATACCCA GCACTCATGG AAGACCA.CT
OS25    TACGGACTGG AACAGTACGA ...ATACCCA GCACTCATGG AAGACCA.CT
MS51    TACGGACTGG AACAGTACGA ...GTACCCA GCACTCATGG AAGACCA.CT
OS37    TACGGACTCG AACAGTACGA ...CACCCCA GCTCTCCTGG AAGACCA.CT
OS70    TACGGACTCG AACAGTACGA ...CACCCCA GCTCTCCTGG AAGACCA.CT
BSS12   TACGGACTCG AACAGTACGA ...GTACCCT GCACTTCTCG AGGACCA.CT
BSS50   TACGGTATCG AGACCTACGA GAAATTCCCA ACAGCCCTTG AAGACCA.CT
BSS65   TACGGTATCG AGACCTACGA GAAATTCCCA ACAGCCCTTG AAGACCA.CT
MS22    TACGGTATCG AGACCTACGA AAAGTTCCCG ACTGCCCTTG AAGACCA.CT
MS42    TACGGTATCG AGACCTACGA GAAATTCCCA ACTGCCCTTG AAGACCA.CT
BSS21   TACGGTATCG AGACTTACGA GAAGTTCCCG ACTGCCCTTG AAGGCCA.CT
BSS8    TACAGCATGG AGCAGTACGA GAAATTCCCG ACCTTAATGG AAGATCA.CT
BSS9    TACAGCATGG AGCAGTACGA GAAATTCCCG ACCTTAATGG AAGATCA.CT
MS16    AATGCAATGG AACAGTACGA ACAGTTCCCA ACCCTTATGG AAGACCA.CT
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MS37    AATGCAATGG AACAGTACGA ACAGTTCCCA ACCCTTATGG AAGACCA.CT
MS23    AATGCAATGG AACAGTACGA ACAGTTCCCA ACCCTTATGG AAGACCA.CT
OS48    AATGCAATGG AACAGTACGA ACAGTTCCCA ACCCTTATGG AAGACCA.CT
OS27    TACGGTATGG AACAGTATGA ACAGTTCCCA ACCATGATGG AAGACCA.CT
OS71    TACGGTATGG AACAGTATGA ACAGTTCCCA ACCATGATGG AAGACCA.CT
OS58    TACGGTATGG AACAGTATGA ACAGTTCCCG ACCATGATGG AAGACCA.CT
OS65    TACGGTATGG AACAGTATGA ACAGTTCCCG ACCATGATGG AAGACCA.CT
PL3     TACGGTATGG AACAGTATGA ACAGTTCCCG ACCATGATGG AAGACCA.CT
HL110   TACGGTATGG AACAGTATGA ACAGTTCCCG ACCATGATGG AAGACCA.CT
OS41    TACGGTATGG AGCAGTATGA ACAGTTCCCG ACCATGATGG AAGACCA.CT
OS110   TACGGTATGG AGCAGTATGA ACAGTTCCCG ACCATGATGG AAGACCA.CT
PL126   TACGGTATGG AGCAGTATGA ACAGTTCCCG ACCATGATGG AAGACCA.CT
OS15    TACGGTATGG AACAGTATGA ACAGTTCCCG ACAATGCTCG AAGACCA.CT
OS20    TACGGTATGG AACAGTATGA ACAGTTCCCG ACAATGCTCG AAGACCA.CT
OS59    TACGGTATGG AACAGTATGA ACAGTTCCCG ACAATGCTCG AAGACCA.CT
OS80    TACGGTATGG AACAGTATGA ACAGTTCCCG ACAATGCTCG AAGACCA.CT
HL99    TACGGTATGG AACAGTATGA ACAGTTCCCG ACAATGCTCG AAGACCA.CT
OS63    TACGGTATGG AGCAGTATGA ACAGTTCCCG ACAATGCTCG AAGACCA.CT
HL34    TACGGTATGG AACAGTATGA ACAGTTCCCG ACGATGCTTG AAGATCA.CT
BSD28   AACGGCATGG AGCAGTATGA GCAGTACCCG ACAATGATGG AAGACCA.CT
PL109   TACGGTATGG AGCAGTACGA AGAGTTCCCG ACCACCCTCG AAAGCCA.CT
PL187   TACGGTATGG AGCAGTACGA AGAGTTCCCG ACCACCCTCG AGAGCCA.CT
PL21    TATGGTATGG AACAGTATGA AGAGTTCCCG ACCACTCTCG AGAGCCA.CT
PL206   TACGGTATGG AACAGTACGA ACAGTTCCCG ACCATGATGG AAGACCA.CT
PL40    TACGGTATGG AACAGTACGA ACAGTTCCCG ACCATGATGG AAGACCA.CT
BSS14   TACGGTATGG AACAGTACGA ACAGTTCCCG ACCATGATGG AAGACCA.TT
BSS43   TACGGTATGG AACAGTACGA ACAGTTCCCG ACCATGATGG AAGACCA.CT
BSD29   AACGGTATGG AACAGTATGA ACAGTTCCCG ACCATGATGG AAGACCA.CT
BSD63   AACGGTATGG AACAGTATGA ACAGTTCCCG ACCATGATGG AAGACCA.CT
BSD95   AACGCCATGG AGCAGTACGA GCAGTTCCCG ACCATGATGG AAGACCA.CT
BSS46   AACGCCATGG AGCAGTACGA GATGTTCCCG ACCATGATGG AGGACCA.CT
BSD21   AACGCCATGG AGCAGTACGA GCAGTTCCCG ACCATGATGG AAGACCA.CT
BSD42   AACGCCATGG AGCAGTACGA GCAGTTCCCG ACCATGATGG AGGACCA.CT
BSD14   AACGCCATGG AGCAGTACGA GCAGTTCCCG ACCATGATGG AGGACCA.CT
BSD67   AACGGCATGG AGCAGTACGA GCAGTACCCG ACCATGATGG AAGACCA.CT
BSD73   AACGGCATGG AGCAGTACGA GCAGTACCCG ACCATGATGG AAGACCA.CT
BSD90   AACGGCATGG AGCAGTACGA GCAGTACCCG ACCATGATGG AGGACCA.CT
HL81    AACGCTATGG AGCAGTACGA GCAGTTCCCG ACCATGATGG AGGACCA.CT
PL240   AACGCCATGG AGCAGTACGA ACAGTTCCCG ACCATGATGG AGGACCA.CT
PL22    AACGCCATGG AGCAGTACGA ACAGTTCCCG ACCATGATGG AGGACCA.CT
BSD61   AACGCCATGG AGCAGTACGA GATGTTCCCG ACCATGATGG AGGACCA.CT
MS61    AACGCCATGG AACAGTACGA GCAGTACCCG ACCATGATGG AAGATCA.CT
BSS52   TACGGTATGG AGCAGTACGA GGAGTTCCCG ACCACGCTCG AGTCCCA.CT
BSS59   TACGGTATGG AGCAGTACGA GGAGTTCCCG ACCACGCTCG AGTCCCA.CT
BSS74   TACGGTATGG AGCAGTACGA GGAGTTCCCG ACCACGCTCG AGTCCCA.CT
PL145   TACGGTATGG AGCAGTACGA ACAGTTCCCG ACCACGCTCG AGAGCCA.CT
PL181   TACGGTATGG AGCAGTACGA ACAGTTCCCG ACCACGCTCG AGAGCCA.CT
HL108   TACGGTATGG AACAGTACGA ACAGTTCCCC ACCACCCTCG AGAGCCA.CT
PL234   TACGGTATGG AACAGTACGA ACAGTTCCCG ACCACCCTCG AGAGCCA.CT
PL1     TACGGTATGG AACAGTACGA ACAGTTCCCC ACCACCCTCG AGAGCCA.CT
PL36    TACGGTATGG AACAGTACGA ACAGTACCCG ACTACCCTCG AGAGCCA.CT
PL238   TACGGTATGG AACAGTACGA ACAGTACCCC ACCACCCTCG AGAGCCA.CT
PL7     TACGGTATGG AACAGTACGA ACAGTTCCCG ACCACCCTCG AGAGCCA.CT
PL43    TACGGTATGG AACAGTACGA ACAGTTCCCG ACCACCCTCG AGAGCCA.CT
PL225   TACGGTATGG AACAGTACGA ACAGTTCCCG ACCACCCTTG AGGACCA.CT
OS77    AACGCCATGG AGCAGTACGA GCAGTTCCCA ACCATGATGG AGGACCA.CT
OS18    TACGCCCTCG AGATGTACGA GAGGTACCCC GCCGCAATGG AGGCCCA.CT
OS55    TACGCTCTGG AGATGTATGA GAAGTACCCC GCCGTCATGG AGGCCCA.CT
HL74    TATGCCCTTG AGACATACGA GAAGTACCCG GCTGCGATGG AGACACA.CT
BSD43   TACGGTATGG AGCAGTATGA GGCATTCCCG ACCCTGCTCG AGGATCA.CT
BSD79   TACGGTATGG AGCAGTATGA GGCATTCCCG ACCCTGCTCG AGGATCA.CT
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PL53    TACGGGATTG AGCAGTACGA GTCATTCCCA ACACTGGTTG AGGATCA.CT

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MS19    TCGGTGGTTC ACAGAGGGCT GCTGTAGTTG CTGCAGCATC TGGTTGTTCC
MS26    TCGGTGGTTC ACAGAGGGCT GCTGTAGTTG CTGCAGCATC TGGTTGTTCC
OS111   TCGGTGGTTC ACAGAGGGCT GCTGTAGTTG CTGCAGCATC TGGTTGTTCC
OS61    TCGGTGGTTC ACAGAGGGCT GCTGTAGTTG CTGCAGCATC TGGTTGTTCC
MS6     TCGGTGGTTC ACAGAGGGCT GCTGTAGTTG CTGCAGCATC TGGTTGTTCC
BSS2    TCGGTGGTTC ACAGAGGGCT GCTGTAGTTG CTGCAGCATC TGGTTGTTCC
BSS49   TCGGTGGATC ACAGAGAGCA TCTATTGTAG CAGCCGCATC TGGTTGTTCC
BSS54   TCGGTGGATC ACAGAGAGCA TCTATTGTAG CAGCCGCATC TGGTTGTTCC
OS105   TCGGTGGATC ACAGAGGGCA TCCATTGTTG CAGCAGCAGC TGGATGTTCA
OS108   TCGGTGGATC ACAGAGGGCA TCCATTGTTG CAGCAGCAGC CGGATGTTCA
OS82    TTGGTGGATC ACAGAGGGCA TCTATTGTTG CAGCAGCAGC AGGATGTTCA
BSS22   TCGGTGGATC CCAGAGGACT GCAGTTGTTT CTGCTGCTGC CGGATGTTCC
BSS26   TCGGTGGATC CCAGAGGACT GCAGTTGTTT CTGCTGCTGC CGGATGTTCC
MS28    TCGGTGGATC CCAGAGGACT GCAGTTGTTT CTGCTGCTGC CGGATGTTCC
OS102   TCGGTGGATC CCAGAGGACT GCAGTTGTTT CTGCTGCTGC CGGATGTTCC
OS25    TCGGTGGATC CCAGAGGACT GCAGTTGTTT CTGCTGCTGC CGGATGTTCC
MS51    TCGGTGGATC CCAGAGGACT GCAGTTGTTT CTGCTGCTGC CGGATGTTCC
OS37    TCGGAGGTTC ACAGAGAGCC GCAGTTGTTT CAGCTGCTGC AGGATGTTCC
OS70    TCGGAGGTTC ACAGAGAGCC GCAGTTGTTT CAGCTGCTGC AGGATGTTCC
BSS12   TCGGAGGTTC CCAGAGGACA GCAGTAATTT CAGCAGCAGC TGGATGTTCA
BSS50   TCGGTGGATC CCAGAGAGCA ACCGTGCTCG CAGCTGCAGC CGGTGTCGCA
BSS65   TCGGTGGATC CCAGAGAGCA ACCGTGCTCG CAGCTGCAGC CGGTGTCGCA
MS22    TCGGTGGATC CCAGAGAGCA ACCGTGCTCG CAGCTGCAGC CGGTGTCGCA
MS42    TCGGTGGATC CCAGAGAGCA ACCGTGCTCG CAGCTGCAGC CGGGGTCGCA
BSS21   TCGGTGGATC CCAGAGAGCA ACCGTGCTCG CAGCTGCAGC CGGTGTCGCA
BSS8    TTGGCGGTTC TCAGCGGGCA GCGGTCATGG CAGCAGCAAG CGGGATTACC
BSS9    TTGGCGGTTC TCAGCGGGCA GCGGTCATGG CAGCAGCAAG CGGGATTACC
MS16    TCGGAGGTTC CCAGCGTGCA GGAGTTATCG CAGCAGCATC CGGTCTCTCA
MS37    TCGGAGGTTC CCAGCGTGCA GGAGTTATCG CAGCAGCATC CGGTCTCTCA
MS23    TCGGAGGTTC CCAGCGTGCA GGAGTTATCG CAGCAGCATC CGGTCTCTCA
OS48    TCGGAGGTTC CCAGCGTGCA GCAGTTCTTG CAGCAGCATC CGGTATCACC
OS27    TCGGCGGTTC CCAGCGTGCA GCCGTTCTTG CAGCAGCATC CGGTATCACC
OS71    TCGGCGGTTC CCAGCGTGCA GCCGTTCTTG CAGCAGCATC CGGTATCACC
OS58    TCGGTGGATC CCAGCGTGCC TCTGTTCTTG CAGCCGCATC CGGTATCACC
OS65    TCGGTGGATC CCAGCGTGCA TCTGTTCTTG CAGCCGCATC CGGTATCACC
PL3     TCGGCGGTTC CCAGCGTGCG TCCGTTCTTG CAGCAGCATC CGGTATCACC
HL110   TCGGCGGTTC CCAGCGTGCA GCCGTTCTTG CAGCAGCCTC CGGTATCTCC
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OS110   TCGGCGGTTC CCAGCGTGCA GCAGTTCTTG CAGCAGCATC CGGTATCACC
PL126   TCGGCGGTTC CCAGCGTGCA GCCGTTCTTG CAGCAGCATC CGGTATCACC
OS15    TCGGTGGTTC ACAGCGTGCA TCCGTCCTTG CAGCAGCAAG CGGTATCACC
OS20    TCGGTGGTTC ACAGCGTGCA TCCGTCCTTG CAGCAGCAAG CGGTATCACC
OS59    TCGGTGGTTC ACAGCGTGCA TCCGTCCTTG CAGCAGCAAG CGGTATCACC
OS80    TCGGTGGTTC ACAGCGTGCA TCCGTTCTTG CAGCAGCAAG CGGTATCACC
HL99    TCGGTGGTTC ACAGCGTGCA GCAGTTCTCG GTGCAGCATG TGGTCTCACG
OS63    TCGGTGGTTC ACAGCGTGCA GCAGTTCTCG GTGCAGCATG TGGTCTCACG
HL34    TCGGCGGTTC CCAGCGTGCA GCCGTTCTTG GAGCGGCAAG CGGTATCACC
BSD28   TCGGCGGTTC ACAGCGTGCC GGTGTTCTTG CCGCTGCCTG CGGTCTGTCG
PL109   TCGGCGGCTC CCAGCGTGCA GGTGTCCTTG CAGCAGCATC AGGTCTTACC
PL187   TCGGCGGTTC CCAGCGTGCA GGTGTCCTTG CAGCAGCATC AGGTCTTACC
PL21    TCGGAGGCTC CCAGCGTGCC GGTGTCCTTG CCGCTGCATC GGGTCTGACC
PL206   TCGGCGGCTC CCAGCGTGCC GGCGTCCTTG CTGCAGCCGC AGGTCTGACC
PL40    TCGGCGGCTC CCAGCGTGCA GGTGTCCTCG CAGCAGCCGC AGGTCTGACC
BSS14   TCGGTGGTTC CCAGCGTGCA GGTGTCCTCG CAGCAGCATC CGGTCTTACC
BSS43   TCGGCGGCTC CCAGCGTGCC GGTGTCCTCG CAGCAGCATC CGGTCTTACC
BSD29   TCGGCGGTTC CCAGCGTGCA GCAGTTCTTG CCGCAGCATC CGGTATCTCG
BSD63   TCGGAGGTTC CCAGCGTGCA GCAGTTCTTG CCGCAGCATC CGGTATCTCT
BSD95   TCGGCGGGTC CCAGCGTGCT GGTGTCATCG CCGCTGCGTC CGGTCTCACG
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BSS46   TCGGCGGTTC CCAGCGTGCC GGTGTCATCG CCGCTGCGTC CGGTCTCACG
BSD21   TCGGCGGGTC CCAGCGTGCT GGTGTCATCG CCGCTGCGTC CGGTCTCACG
BSD42   TCGGCGGTTC CCAGCGTGCC GGTGTCATCG CCGCTGCGTC CGGTCTCACG
BSD14   TCGGCGGGTC CCAGCGTGCC GGTGTCATCG CCGCTGCGTC CGGTCTCACG
BSD67   TCGGCGGGTC CCAGCGTGCC GGTGTCATCG CCGCTGCGTC CGGTCTCACG
BSD73   TCGGCGGGTC CCAGCGTGCC GGTGTGCTTG CCGCCGCCTG CGGTCTGTCG
BSD90   TCGGCGGTTC CCAGCGTGCC GGTGTGCTTG CCGCCGCCTG CGGTCTGTCG
HL81    TCGGCGGGTC CCAGCGTGCC GGTGTCATCG CCGCCGCATC CGGTCTCACG
PL240   TCGGCGGATC CCAGCGTGCC GGTGTCATCG CCGCCGCGTC CGGTCTGACG
PL22    TGGGCGGGTC CCAGCGTGCC GGTGTCATCG CCGCCGCGTC CGGTCTGACA
BSD61   TCGGCGGTTC CCAGCGTGCC GGTGTCATCG CCGCTGCGTC CGGTCTGACG
MS61    TCGGCGGGTC CCAGCGTGCC GGTGTCATCG CCGCCGCATC CGGTCTCACG
BSS52   TCGGCGGCTC CCAGCGTGCA ACCGTCCTCG CCGCAGCATC TGGTGTCTCA
BSS59   TCGGCGGCTC CCAGCGTGCA ACCGTCCTCG CCGCAGCATC TGGTGTCTCA
BSS74   TCGGCGGCTC CCAGCGTGCA ACCGTCCTCG CCGCAGCATC TGGTGTCTCG
PL145   TCGGCGGTTC ACAGCGTGCA TCCGTCCTCG CAGCAGCGTC CGGTATCTCC
PL181   TCGGCGGTTC ACAGCGTGCA TCCGTCCTCG CAGCAGCGTC CGGTATCTCC
HL108   TCGGAGGTTC CCAGCGTGCA TCCGTCCTTG CAGCAGCATC AGGTATCTCC
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PL1     TTGGCGGTTC CCAGCGTGCA TCCGTCCTTG CAGCAGCATC AGGTATCTCC
PL36    TCGGCGGTTC CCAGCGTGCA TCCGTCCTTG CAGCTGCATC AGGTATCTCC
PL238   TCGGCGGTTC GCAGCGTGCA TCCGTCCTTG CAGCAGCATC AGGTATCTCC
PL7     TTGGCGGTTC CCAGCGTGCA TCCGTCCTTG CAGCAGCATC AGGTATCTCC
PL43    TTGGCGGATC CCAGCGTGCA TCCGTCCTTA CAGCAGCATC AGGTATCTCC
PL225   TCGGCGGTTC CCAGCGGGCA TCCGTCCTTG CAGCAGCATC CGGTATCTCC
OS77    TCGGCGGTTC CCAGCGTGCT GGTGTCATTG CAGCTGCGTC AGGTTTGACC
OS18    TCGGTGGATC CCAGAGGGCG ACCGTCGCAG CCGCGGCATC CGGTATCGCC
OS55    TCGGTGGATC CCAGAGGGCA ACGGTTGCCG CTGCGGCATC CGGTATCGCC
HL74    TCGGTGGTTC TCAGAGAGCA ACCGTTGCCG CTGCTTCGAC CGGTATCGCC
BSD43   TCGGTGGATC CCAGAGGGCT TCCGTTCTGG CAGCAGCATC CGGTATCACC
BSD79   TCGGTGGATC CCAGAGGGCT TCCGTTCTGG CAGCAGCATC CGGTATCACC
PL53    TCGGCGGATC CCAGAGGGCA TCAGTCCTCG CTGCCTGCTC CGGTATTGGA

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MS26    ACAGGTTTCG CAACTGGAAA CGGTCAAGCT GGTCTAAGCG CATGGTATCT
OS111   ACAGGTTTCG CAACTGGAAA CGGTCAAGCT GGTCTAAGCG CATGGTATCT
OS61    ACAGGTTTCG CAACTGGAAA CGGTCAAGCT GGTCTAAGCG CATGGTATCT
MS6     ACAGGTTTCG CAACTGGAAA CGGTCAAGCT GGTCTAAGCG CATGGTATCT
BSS2    ACAGGTTTCG CAACTGGAAA CGGTCAAGCT GGTCTAAGCG CATGGTACCT
BSS49   ATTGCATTTG CAACTGGAAA CGCTCAGACC GGTTTAAGCG GATGGTACCT
BSS54   ACTGCATTTG CAACTGGAAA CGCTCAGACC GGTTTAAGCG GATGGTACCT
OS105   ACTGCATTCG CTACTGGAAA TGCTCAGACC GGTCTAAGTG GATGGTACTT
OS108   ACTGCATTCG CTACTGGAAA TGCTCAGACC GGTCTAAGTG GATGGTACTT
OS82    ACTGGATTCG CTACAGGAAA CTCACAGACT GGACTAAGCG CATGGTACCT
BSS22   GTTGCCTTTG CAACCGGAAA CTCCAACGCA GGAATTAACG GATGGTACTT
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MS51    GTTGCCTTTG CAACCGGAAA CTCCAACGCA GGAATTAACG GATGGTACTT
OS37    GTTGCCTTTG CAACCGGAAA CTCCAACGCA GGAATCAACG GATGGTACTT
OS70    GTTGCCTTTG CAACCGGAAA CTCCAACGCA GGAATCAACG GATGGTACTT
BSS12   TGTGCATTTG CAACTGCAAA CTCCAATGCA GGAATCAACG GATGGTACTT
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BSS65   TGTGCACTCG GAACTGCAAA CGCAAACGCC GGTCTCTCAG GCTGGTACCT
MS22    GTCGCACTCG GAACTGCAAA CGCAAATGCC GGTCTCTCAG GCTGGTACCT
MS42    GTTGCACTCG GAACTGCAAA CGCAAACGCT GGTCTTTCAG GCTGGTACCT
BSS21   ACTGCCCTTG CAACTGCAAA CGCAAATGCT GGTCTCTCTG GCTGGTACCT
BSS8    ACAGCGATCG GGACCGGGAA CTCCAATGCC GGTCTCAATG CATGGTACCT
BSS9    ACAGCGATCG GGACCGGGAA CTCCAATGCC GGTCTCAATG CATGGTACCT
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MS23    ACCGCAATTG CAACTGCAAA CTCCAACGCC GGTCTGAATG GATGGTACCT
OS48    ACTTCCATTG CAACCGGAAA CTCCAACGCC GGTCTCAACG GCTGGTATCT
OS27    ACGTCAATTG CAACCTCCAA CTCCAACGCC GGTCTCAACG CCTGGTATCT
OS71    ACGTCAATTG CAACCTCCAA CTCCAACGCC GGTCTCAACG GCTGGTATCT
OS58    ACGTCAATTG CAACCTCCAA CTCGAACGCC GGTCTCAACG CCTGGTATCT
OS65    ACGTCAATTG CAACGGCAAA CTCGAACGCC GGTCTCAACG CCTGGTATCT
PL3     ACGTCCATTG CAACGGCAAA CTCCAACGCC GGTCTCAACG GCTGGTATCT
HL110   ACGTCGATTG CAACCGGAAA CTCCAACGCC GGTCTCAACG GCTGGTATCT
OS41    ACGTCGATTG CAACCGGAAA CTCCAACGCC GGTCTCAACG GCTGGTATCT
OS110   ACTTCCATTG CAACCGGAAA CTCCAACGCC GGTCTCAACG GCTGGTATCT
PL126   ACCTCCATTA CAACAGGAAA CTCCAACGCC GGTCTCAATG GCTGGTATCT
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OS20    ACTTCCATTG CAACCGGAAA CTCCAATGCC GGTCTGAATG CATGGTATCT
OS59    ACTTCCATTG CAACCGGAAA CTCCAATGCC GGTCTGAATG CATGGTATCT
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OS63    ACTTCCATTG CAACCGGAAA CTCCAATGCC GGTCTGAATG GCTGGTATCT
HL34    ACTTCCATTG CAACCGGAAA TTCCAACGCC GGTCTGAACG GCTGGTATCT
BSD28   TGCTCAATTG CAACAGGAAA CTCCAATGCA GGTCTTAACG GCTGGTATCT
PL109   TGTGCAATTG CAACCGCCAA CTCGAACGCT GGCTTAAACG GCTGGTACAT
PL187   TGTGCAATTG CAACCGCCAA CTCGAACGCT GGCTTAAACG GCTGGTACAT
PL21    TGTGCAATTG CAACCGCCAA CTCCAACGCT GGCCTGAACG GATGGTATAT
PL206   ACCTCGATTG CAACGGCCAA CTCCAACGCC GGTCTGAACG GCTGGTACAT
PL40    ACCTCGATTG CAACGGCCAA CTCCAACGCC GGTCTCAACG GCTGGTACAT
BSS14   ACTGCAATTG CAACCGGTAA CTCCAACGCC GGTCTCAACG GCTGGTACAT
BSS43   ACGGCAATCG CCACTGGTAA CTCCAACGCC GGTCTCAACG GCTGGTACAT
BSD29   ACCGCCATTG CAACGGGAAA CTCAAACGCT GGCCTGAACG GCTGGTACCT
BSD63   ACCGCCATTG CAACGGCAAA CTCAAACGCT GGTCTGAACG GCTGGTACCT
BSD95   ACCGCCATCG CAACCGGCAA CTCCAACGCC GGTCTCAACG GATGGTATCT
BSS46   ACCGCCATCG CAACCGGCAA CTCCAACGCC GGTCTCAACG GATGGTATCT
BSD21   ACCGCCATCG CAACCGGCAA CTCGAACGCC GGTCTCAACG GATGGTATCT
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BSD73   ACCTCCATCG CGACCGGAAA CTCGAACGCC GGCCTGAACG CCTGGTACCT
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HL81    ACCTCTATCA CGACCGGAAA CTCGAACGCC GGCTTAAACG GCTGGTATCT
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PL22    ACCTTTATCA CGACCGGCAA CTCCAACGCC GGCCTGAACG CCTGGTACCT
BSD61   ACCTCTATCG CGACCGGCAA CTCGAACGCC GGCCTGAACG CCTGGTACCT
MS61    ACCGCCATTG CAACCGGAAA CTCCAACGCC GGTCTGAACG CCTGGTACCT
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BSS59   ACGGCACTCG CCACGGCAAA CTCCAACGCT GGTCTGAACG GCTGGTACAT
BSS74   ACGGCACTCG CCACGGCAAA CTCCAACGCT GGTCTGAACG GCTGGTACAT
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OS18    GGATCCATGG CAACCGGTAA CGCCGACTGC GGTGTCAACA TGTGGTATCT
OS55    GGAGCCATGG CAACCGGTAA CGCCGACTGC GGTGTCAACA TGTGGTACCT
HL74    GGTTCAATGG CAACCGGTAT CGCTGATTGT GGTCTAAATC TCTGGTATCT
BSD43   TCCGCCATCG CCTCTGGCCA CAGCCAGGTA GGCCTTGCCG GCTGGTACCT
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PL53    TGCGGCCTGG CAACCGGCCA CTCCCAGATC GGCCTGGCTG GCTGGTACCT

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MS26    GTCCATGTAC TTACAC.AAA GAACAGCACA GCCGACTTGG TTTCTACGGT
OS111   GTCCATGTAC TTACAC.AAA GAACAGCACA GCCGACTTGG TTTCTACGGT
OS61    GTCCATGTAC TTACAC.AAA GAACAGCACA GCCGACTTGG TTTCTACGGT
MS6     GTCCATGTAC TTACAC.AAA GAACAGCACA GCCGACTTGG TTTCTACGGT
BSS2    GTCCATGTAC TTACAC.AAA GAACAGCACA GCCGACTTGG GTTCTACGGT
BSS49   CTCCATGTAC CTGCAC.AAA GAACAGCACA GCAGACTTGG ATTCTACGGT
BSS54   CTCCATGTAC CTGCAC.AAA GAACAGCACA GCAGACTTGG ATTCTACGGT
OS105   ATCCATGTAC TTACAC.AAA GAACAGCACT CCAGACTTGG TTTCTATGGT
OS108   ATCCATGTAC TTACAC.AAA GAACAGCACT CCAGACTTGG TTTCTATGGT
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BSS26   AAGCCAGATC CTGCAC.AAA GAAGCACACA GCAGACTCGG TTTCTACGGT
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OS70    AAGCCAGATC CTACAC.AAA GAAGCACACA GCAGACTCGG TTTCTACGGT
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BSS65   CTCCATGTAC CTGCAC.AAG GAAGCATGGG GCAGACTCGG ATTCTTCGGT
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MS42    CTCCATGTAC CTGCAC.AAG GAAGCATGGG GCAGACTCGG ATTCTTGGGA
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BSS9    TGCGATGATT ATCCAC.AAG GATGCGTGGT CGCGTCTCGG GTTCTTCGGG
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MS37    TTCAATGCTC ATGCAC.AAG GAAGGCTGGT CACGTCTCGG ATTCTTCGGA
MS23    TTCAATGCTC ATGCAC.AAG GAAGGCTGGT CACGTCTCGG ACTCTTCGGA
OS48    GTCCATGCTT CTCCAC.AAA GACGGATGGT CCAGACTTGG TTTCTTCGGC
OS27    GTCCATGCTT ATGCAC.AAG GACGGATGGT CCAGACTTGG TTTCTTCGGC
OS71    GTCCATGCTT ATGCAC.AAG GACGGATGGT CCAGACTTGG TTTCTTCGGC
OS58    GTCCATGCTT ATGCAC.AAG GACGGATGGT CCAGACTTGG TTTCTTCGGC
OS65    GTCCATGCTT ATGCAC.AAG GACGGATGGT CCAGACTTGG TTTCTTCGGC
PL3     GTCCATGCTT ATGCAC.AAG GACGGATGGT CCAGACTTGG TTTCTTCGGC
HL110   GTCCATGCTT CTGCAC.AAA GACGGCTGGT CCAGACTTGG TTTCTTCGGC
OS41    GTCCATGCTT CTGCAC.AAA GACGGCTGGT CCAGACTTGG TTTCTTCGGC
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PL126   GTCCATGCTT CTGCAC.AAA GACGGATGGT CCAGACTTGG TTTCTTCGGC
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OS59    CTCGATGCTC ATGCAC.AAG GACGGCTGGT CACGTCTTGG TTTCTTCGGC
OS80    CTCGATGCTT ATGCAC.AAG GACGGCTGGT CACGTCTTGG TTTCTTCGGC
HL99    GTCCATGCTT ATGCAC.AAG GACGGCTGGT CACGTCTCGG CTTCTTCGGT
OS63    CTCGATGCTT ATGCAC.AAG GACGGCTGGT CACGTCTCGG CTTCTCCGGC
HL34    CTCGATGCTT ATGCAC.AAG GACGGCTGGT CACGTCTTGG TTTCTTCGGC
BSD28   CTCAATGCTC ATGCAC.AAG GAAGGATGGT CACGTCTCGG GTTCTTCGGC
PL109   GTCCATGCTC GCCCAC.AAG GAAGGCTGGT CACGTCTCGG CTTCTTCGGC
PL187   GTCCATGCTC GCCCAC.AAG GAAGGCTGGT CACGTCTCGG CTTCTTCGGC
PL21    GTCCATGCTC GCTCAC.AAG GAGGGCTGGT CACGTCTCGG CTTCTTCGGC
PL206   GTCCATGCTC CTGCAC.AAG GAAGGCTGGT CGCGTCTCGG CTTCTTCGGC
PL40    GTCCATGCTC CTGCAC.AAG GAAGGCTGGT CACGTCTCGG TTTCTTCGGC
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BSS43   GTCCATGCTC CTGCAC.AAG GAAGGCTGGT CACGTCTCGG CTTCTTCGGC
BSD29   CTCCATGCTC CTGCAC.AAT GACGGATGGT CGCGTCTCGG ATTCTTCGGA
BSD63   CTCCATGCTC CTCCAC.AAG GAAGGCTGGT CACGTCTCGG ATTCTTCGGA
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BSS46   CTCGATGCTC CTGCAC.AAG GACGGCTGGT CGCGTCTCGG CTTCTTCGGC
BSD21   CTCGATGCTC CTGCAC.AAG GACGGTTGGT CGCGTCTCGG GTTCTTCGGA
BSD42   CTCCATGCTC CTGCAC.AAG GACGGTTGGT CGCGTCTCGG GTTCTTCGGA
BSD14   CTCGATGCTC CTGCAC.AAG GACGGCTGGT CGCGTCTCGG GTTCTTCGGA
BSD67   CTCGATGCTC CTGCAC.AAG GACGGATGGT CGCGTCTCGG CTTCTTCGGA
BSD73   CTCGATGCTC CTGCAC.AAG GACGGCTGGT CGCGTCTCGG CTTCTTCGGC
BSD90   CTCCATGCTC CTGCAC.AAG GACGGATGGT CGCGTCTCGG CTTCTTCGGC
HL81    CTCCATGCTC CTGCAC.AAG GACGGATGGT CGCGTCTCGG CTTCTTCGGC
PL240   CTCCATGCTC CTGCAC.AAG GACGGATGGT CGCGTCTTGG CTTCTTCGGC
PL22    TTCCATGCTC CTGCAC.AAG GACGGATGGT CGCGTCTTGG TTTTTTCGGC
BSD61   CTGCATGCTC CTGCAC.AAG GACGGTTGGT CGCGTCTCGG CTTCTTCGGA
MS61    CTCCATGCTC CTGCAC.AAG GACGGATGGT CACGTCTCGG CTTCTTCGGC
BSS52   GTCCATGCTC ATGCAC.AAG GAAGCATGGT CACGTCTCGG CTTCTTCGGC
BSS59   GTCCATGCTC ATGCAC.AAG GAAGCATGGT CACGTCTCGG CTTCTTCGGC
BSS74   GTCCATGCTC ATGCAC.AAG GAAGCATGGT CACGTCTCGG CTTCTTCGGC
PL145   GTCGATGCTC ATGCAC.AAG GAAGGCTGGT CACGTCTCGG CTTCTTCGGT
PL181   GTCGATGCTC ATGCAC.AAG GAAGGCTGGT CACGTCTCGG CTTCTTCGGC
HL108   GTCCATGCTC GCCCAC.AAG GAAGGCTGGT CACGTCTCGG CTTCTTCGGC
PL234   GTCCATGCTC GCCCAC.AAG GAAGGCTGGT CACGTCTCGG CTTCTTCGGT
PL1     GTCCATGCTC GCCCAC.AAG GAAGGCTGGT CACGTCTTGG CTTTTTCGGC
PL36    CTCCATCCTC CCCCAC.AAC CAACCCTCCT CACCTCTCCC CTTCTTCCCC
PL238   GTCCATGCTC GCCCCC.AAG GAAGGCTGGT CACGTCTCGG CTTCTTCGGC
PL7     GTCCATGCTC GCCCAC.AAG GAAGGCTGGT CACGTCTCGG TTTTTTCGGC
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PL225   GTCCATGCTC GCCCAC.AAG GAAGGCTGGT CACGTTTCGG CTTCTTCGGC
OS77    CTCGATGCTC ATGCAC.AAG GAAGGATGGT CACGTCTCGG TTTCTTCGGC
OS18    GTCCATGCTG CAGCAC.AAG GAGAGGACTG GAAGGCTCGG ATTCTACGGA
OS55    GTCCATGCTG CAGCAC.AAG GAGAGGACCG GAAGGCTCGG ATTCTACGGA
HL74    GTCTATGCTT CAGCAA.AAG GAGAGGACCG GAAGGCTCGG ATTCTACGGA
BSD43   CTCCATGCTC CTGCAC.AAG GAAGGCTGGG GCAGACTGGG CTTCTTCGGC
BSD79   CTCCATGCTC CTGCAC.AAG GAAGGCTGGG GCAGACTGGG CTTCTTCGGC
PL53    CTCCATGCTG CTGCAC.AAG GAGGCATGGG GCAGACTGGG CTTCTTCGGA

        351                                                 400
MS19    TACGACCTGC AGGACCAGTG TGGTGCATCC AACGTATTCT CCATCCGTGG
MS26    TACGACCTGC AGGACCAGTG TGGTGCATCC AACGTATTCT CCATCCGTGG
OS111   TACGACCTGC AGGACCAGTG TGGTGCATCC AACGTATTCT CCATCCGTGG
OS61    TACGACCTGC AGGACCAGTG TGGTGCATCC AACGTATTCT CCATCCGTGG
MS6     TACGACCTGC AGGACCAGTG TGGTGCATCC AACGTATTCT CCATCCGTGG
BSS2    TACGACCTGC AGGACCAGTG TGGTGCATCC AACGTATACT CCATCCGTGG
BSS49   TACGACCTTC AGGACCAGTG TGGTGCCTCC AACGTGTTCT CAATTAGAGG
BSS54   TACGACCTTC AGGACCAGTG TGGTGCCTCC AACGTGTTCT CAATTAGAGG
OS105   TACGACCTGC AGGATCAGTG TGGTGCATCC AACGTGTTCT CCATCCGTGG
OS108   TACGACCTGC AGGATCAGTG TGGTGCATCC AACGTATTCT CCATCCGTGG
OS82    TACGATCTCC AAGACCAGTG TGGTGCATCC AACGTGTTCT CAATTAGGAA
BSS22   TACGACCTGC AGGACCAGTG TGGAGCATCC AACTCTCTCT CCATCAGGAG
BSS26   TACGACCTGC AGGACCAGTG TGGAGCATCC AACTCTCTCT CCATCAGGAG
MS28    TACGACCTGC AGGACCAGTG TGGAGCATCC AACTCTCTCT CCATCAGGAG
OS102   TACGACCTGC AGGACCAGTG TGGAGCATCC AACTCTCTCT CCATCAGGAG
OS25    TACGACCTGC AGGACCAGTG TGGAGCATCC AACCCTCTCT CCATCAGGAG
MS51    TACGACTTGC ATGACCAGTG TGGAGCATCC AACTCTCTCT CCATCAGGAG
OS37    TACGACCTGC AGGACCAGTG TGGAGCATCC AACTCTCTCT CCATCAGGAG
OS70    TACGACCTGC AGGACCAGTG TGGAGCATCC AACTCTCTCT CCATCAGGAG
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BSS65   TTCGACCTGC AGGATCAGTG TGGTGCCACA AACGTTCTGT CCTACCAGGG
MS22    TACGACCTGC AGGACCAGTG CGGTGCCACA AACGTTCTGT CCTACCAGGG
MS42    TACGACCTGC AGGACCAGTG CGGTGCCACA AACGTTCTGT CCTACCAGGG
BSS21   TACGACCTGC AGGACCAGTG TGGTGCCACA AATGTTCTGT CCTACCAGGG
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OS71    TACGATCTGC AGGACCAGTG CGGTTCCGCA AACTCTCTGT CCATCAGACC
OS58    TACGATCTGC AGGACCAGTG CGGTTCCGCA AACTCTCTGT CCATCAGACC
OS65    TACGATCTGC AGGACCAGTG CGGTTCCGCA AACTCTCTGT CCATCAGACC
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HL110   TACGATCTGC AGGACCAGTG CGGTTCCGCA AACTCACTCT CCATCAGACC
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OS59    TACGATCTGC AGGACCAGTG CGGTTCCGCA AACTCACTCT CCATCAGACC
OS80    TACGACCTGC AGGACCAGTG CGGTTCAGCA AACTCACTCT CCATCAGACC
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HL34    TACGATCTGC AGGACCAGTG CGGTTCCGCA AACTCACTCT CCATCAGACC
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PL187   TACGACCTGC AGGACCAGTG CGGTTCCGCG AACTGTATGG CCATCCGGCC
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PL206   TACGACCTGC AGGACCAGTG CGGTTCCGCA AACTCGCTCT CCGTCCGGCC
PL40    TACGACCTGC AGGACCAGTG CGGTTCCGCA AACTCGCTCT CCGTCCGGCC
BSS14   TACGACCTGC AGGACCAGTG CGGTTCCGCA AACTCGCTCT CCGTCAGGCC
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BSS46   TACGACCTCC AGGACCAGTG CGGGTCTGCA AACTCGCTCT CCATGGAGCC
BSD21   TACGACCTCC ATGACCAGTG CGGTTCTGCA AACTCCCTCT CCATGGGTGC
BSD42   TACGACCTCC AGGACCAGTG CGGTTCTGCA AACTCCCTCT CCATGGAGTC
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PL181   TACGACCTCC AGGACCAGTG CGGTTCCGCG AACTGTATGT CCGTCCGCCC
HL108   TACGACCTGC AGGACCAGTG CGGTTCAACC AACTCAATGT CGATCAGGCC
PL234   TACGACCTGC AGGACCAGTG CGGTTCCGCA AACTCAATGT CCGTCAGGCC
PL1     TACGATCTGC AGGACCAGTG CGGTTCCGCG AACTCCATGT CCGTCCGGCC
PL36    TACGACCTGC AGGACCAGTG CGGTTCCGCG AACTCGATGT CCATCCGGCC
PL238   TACGACCTGC AGGACCAGTG CGGTTCCGCC AACTGTATGG CCATCCGGCC
PL7     TACGACCTGC AGGACCAGTG CGGTTCCGCA AACTCCATGG CCATCCGGCC
PL43    TACGACCTGC AGGACCAGTG CGGTTCCTCG AACTCGCTCT CCGTCAGGCC
PL225   TACGACCTGC AGGACCAGTG CGGTTCCGCA AACTCACTCT CTGTCCGGCC
OS77    TACGACCTGC AGGACCAGTG TGGTTCAGCA AACTCGATGT CGATCAGACC
OS18    TACGACTTGC AGGACCAGTG CGGTTCCGCC AACTCGTTCT CCTACAGGTC
OS55    TACGACTTGC AGGACCAGTG CGGTTCCGCT AACTCGTTCT CCTACAGATC
HL74    TACGACCTTC AGGACCAGTG CGGTTCCGCG AACTCTTTCG CATACAGATC
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BSD79   TACGATCTGC AGGATCAGTG CGGTCCAACC AACGTATTCT CCTACCAGTC
PL53    TACGATCTGC AGGATCAGTG CGGCCCAACC AACGTTTTTT CCTATCAGTC

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MS26    AGACGAAGGA TTACCACTGG AACTACGTGG AGCTAACTAC
OS111   AGACGAAGGA TTACCACTGG AACTACGTGG AGCTAACTAC
OS61    AGACGAAGGA TTACCACTGG AACTACGTGG AGCTAACTAC
MS6     AGACGAAGGA TTACCACTGG AACTACGTAG AGCTAACTAC
BSS2    AGACGAAGGA TTACCACTGG AACTACGTGG AGCTAACTAC
BSS49   AGACGAAGGA TTACCAACTG AACTGAGAGG AGCTAACTAC
BSS54   AGACGAAGGA TTACCAACTG AACTGAGAGG AGCTAACTAC
OS105   AGACGAAGGA TTACCGTCTG AACTGAGAGG AGCTAACTAC
OS108   AGACGAAGGA TTACCAACTG AACTGAGAGG AGCTAACTAC
OS82    CGACGAAGGA TTACCAGTGG AAATGAGAGG ACCAAACTAC
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BSS26   CGACGAAGGT TTAATCCACG AACTACGTGG TCCTAACTAC
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OS102   CGACGAAGGT TTAATCCACG AACTACGTGG TCCTAACTAC
OS25    CGACGAAGGT TTAATCCACG AACTATGTGG TCCTAACTAC
MS51    CGACGAAGGT TTAATCCACG AACTACGTGG TCCTAACTAC
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OS70    CGACGAAGGT TTAATCCACG AACTACGTGG TCCTAACTAC
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BSS50   GGACGAAGGT CTCCCAGACG AACTCCGTGG GCCAAACTAT
BSS65   CGACGAAGGT CTCCCAGACG AACTCCGTGG TCCAAACTAC
MS22    CGACGAAGGT CTCCCAGACG AACTCCGTGG TCCAAACTAC
MS42    GGACGAAGGT CTCCCAGACG AACTCCGCGG TCCAAACTAC
BSS21   TGACGAAGGT CTCCCAGACG AACTCCGTGG TCCAAACTAC
BSS8    AGACCAGGGT GCGATCCGCG AAGGCCGCGG GCCGAACTAT
BSS9    TGACCGCGGA GCAATGGGCG AATTAAGAGG CCCCAACTAC
MS16    TGACGAGGGA TGTATTGGTG AATACCGTGG TCCAAACTAC
MS37    TGACGAGGGA TGTATTGGTG AATACCGTGG TCCAAACTAC
MS23    TGACGAGGGA TGTATTGGTG AATACCGTGG TCCAAACTAC
OS48    CGACGAGGGC TGTATCGGCG AGTTCCGTGG ACCGAACTAC
OS27    TGATGAAGGC TGTATCGGCG AGTTCCGTGG ACCAAACTAC
OS71    TGATGAAGGC TGTATCGGCG AGTTCCGTGG ACCAAACTAC
OS58    TGATGAAGGC TGTATCGGCG AGTTCCGTGG ACCAAACTAC
OS65    TGATGAAGGC TGTATCGGCG AGTTCCGTGG ACCAAACTAC
PL3     TGATGAAGGC TGTATCGGCG GGTTCCGAGG ACCAAACTAC
HL110   TGATGAAGGC TGTATCGGCG AGTTCCGCGG ACCAAACTAC
OS41    TGATGAAGGC TGTATCGGCG AGTTCCGCGG ACCAAACTAC
OS110   CGACGAAGGC TGTATCGGCG AGTTCCGTGG ACCGAACTAC
PL126   CGACGAGGGC TGTATCGGCG AGTTCCGTGG ACCGAACTAC
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OS20    TGATGAAGGC TGTATCGGCG AGTTCCGTGG ACCGAACTAC
OS59    TGATGAAGGC TGTATTGGCG AGTTCCGTGG ACCAAACTAC
OS80    TGATGAAGGC TGTATCGGCG AGTTCCGTGG ACCAAACTAC
HL99    TGATGAAGGC TGTATCGGCG AGTTCCGCGG ACCAAACTAC
OS63    TGATGAAGGC TGTATCGGCG AGTTCCGTGG ACCGAACTAC
HL34    TGATGAAGGT TGTATCGGAG AATTCCGTGG ACCGAACTAC
BSD28   TGACCGCGGT CTGATGGGAG AACTGCCTGG GCCAAACTAC
PL109   CGACGAGGGT TGTATCGGCG AACTCCGCGG ACCGAACTAC
PL187   CGACGAGGGC TGTATCGGCG AACTCCGTGG ACCGAACTAC
PL21    CGATGAGGGC TGTGTCGGTG AACTCCGTGG CCCGAACTAC
PL206   TGACGAAGGT TGTGTCGGTG AGCTCCGTGG CCCGAACTAT
PL40    TGACGAAGGT TGTGTCGGCG AGCTCCGTGG CCCGAACTAC
BSS14   TGACGAAGGT TGTGTCGGTG AACTCCGTGG CCCGAACTAT
BSS43   TGACGAAGGT TGTGTCGGTG AACTCCGTGG CCCGAACTAC
BSD29   CGACCGCGGT CTGATGGGAG AACTGCCTGG GCCGAACTAT
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BSD63   TGACGAAGGC TGTATCGGTG AGTTCCGTGG CCCGAACTAC
BSD95   CGACCGCGGT CTAATGGGCG AGCTGCGTGG TCCGAACTAC
BSS46   CGACCGCGGT CTAATGGGCG AGCTGCGTGG CCCGAACTAC
BSD21   CGACCGCGGT CTGATTCGCG AACTGCCTGG TCCGAACTAT
BSD42   CGACCGCGGT CTGATTGGCG AACTGCCTGG GCCGAACTAT
BSD14   CGACCAAGGT GCTATCCGCG AGCTGCCTGG TCCGAACTAT
BSD67   AGACCAAGGC GCGATCCGCG AGGTCCGTGG TCCGAACTAC
BSD73   CGACCGCGGT CTAATGGGCG AGCTGCGTGG TCCGAACTAC
BSD90   CGACCGCGGT CTGATGGGCG AACTGCGTGG CCCGAACTAC
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PL240   CGACCGCGGC CTGGTGGGAG AACTCCGTGG CCCGAACTAC
PL22    CGACCGCGGC CTGATGGGAG AACTCCGTGG CCCGAACTAC
BSD61   CGACCGCGGT CTGATGGGCG AACTGCGTGG CCCGAACTAT
MS61    CGACCGCGGC CTGATGGGAG AACTCCGTGG CCCGAACTAC
BSS52   CGACGAGGGT CTGCTCGGAG AGCTCCGTGG ACCGAACTAC
BSS59   CGACGAGGGT CTGCTCGGAG AGCTCCGTGG TCCGAACTAC
BSS74   CGACGAGGGT CTGCTCGGAG AGCTCCGTGG ACCGAACTAT
PL145   TGACGAGGGC TGTGTCGGTG AGCTCCGTGG CCCGAACTAC
PL181   AGACGAAGGC TGTATCGGCG AGCTTCGTGG ACCCAACTAC
HL108   CGACGAGGGC TGCATCGGCG AGCTCCGTGG ACCCAACTAC
PL234   CGACGAAGGC TGTATCGGCG AACTCCGTGG ACCAAACTAC
PL1     CGACGAAGGC TGTATGGGTG AACTCCGCGG ACCCAACTAC
PL36    TGACGAAGGC TGTATCGGCG AACTCCGTGG CCCGAACTAC
PL238   CGACGAGGGT TGTATCGGCG AACTCCGCGG ACCGAACTAC
PL7     TGACGAAGGC TGTATCGGCG AACTCCGTGG ACCGAACTAC
PL43    TGATGAAGGC TGTGTAGGTG AACTCCGTGG ACCCAACTAC
PL225   TGACGAGGGC TGTGTCGGTG AGCTCCGCGG CCCGAACTAC
OS77    GGATGAAGGT CTCCTCGGTG AACTCCGTGG ACCAAACTAC
OS18    CGACGAAGGT CTCCCCATGG AGCTGAGAGG TCCCAACTAC
OS55    CGACGAGGGT CTCCCCATGG AGCTGAGAGG TCCCAACTAC
HL74    TGATGAAGGT CTGCCTATGG AGCTCAGAGG ACCTAACTAC
BSD43   AGACGAGGGC AACCCACTCG AGCTGAGGGG AGCTAACTAC
BSD79   AGACGAGGGC AACCCACTCG AGCTGAGGGG AGCTAACTAC
PL53    AGACGAGTCC AACCCACTCG AGCTGAGGGG CGCCAACTAT
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APPENDIX B.

Alignment of predicted amino acid sequences determined in this study for *mcrA* PCR products from landfill samples.

Landfill samples from which *mcrA* sequences were amplified:

MS Excavated refuse sample from Mucking site.

OS Excavated refuse sample from Odcombe site.

BSD Excavated refuse sample from Brogborough site, depth 18m.

BSS Excavated refuse sample from Brogborough site, depth 3m.

PL Leachate sample from Poyle site.

HL Leachate sample from Hermitage site.

The alignment was created using PILEUP (GCG Wisconsin Package 10.1, Genetics Computer Group, Wisconsin, USA).

Gap creation penalty: 8

Gap extension penalty: 2

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          1                                                  50
OS37      YTDDILDDFV YYGMEYVDDK YG-------- ICGTKATTEV VHDIAAEVTM
OS70      YTDDILDDFL YYGMEYVDGK YG-------- ICGTKATTEV VHDIAAEVTM
BSS22     YTDDILDDFV YYGMEYVDDK YG-------- ICGTKATTEV VHDIASEVTM
BSS26     YTDDILDDFV YYGMEYVDDK YG-------- ICGTKATTEV VHDIASEVTM
MS28      YTDDILDDFV YYGMEYVDDK YG-------- ICGTKATTEV VHDIASEVTM
MS51      YTDDILDDFV YYGMEYVDDK YG-------- ICGTKATTEV VHDIASEVTM
OS102     YTDDILDDFV YYGMEYVDDT YG-------- ICGTKATTEV VHDIASEVTM
OS25      YTDDILDDFV YYGMEYVDDK YG-------- ICGTKATTEV VHDIASEVTM
BSS12     YTDDILDDFL YYGKEYIEDK YG-------- MCGAKPSMDV VKDIASEVTL
MS19      YTDNILDDFT YYGKEYVEDK YGG------- LTEAPNNMDT ILDVASEVTF
MS26      YTDNILDDFT YYGKEYVEDK YGG------- LTEAPNNMDT ILDVASEVTF
OS111     YTDNILDDFT YYGKEYVEDK YGG------- LTEAPNNMDT ILDVASEVTF
OS61      YTDNILDDFT YYGKEYVEDK YGG------- LTEAPNNMDT ILDVASEVTF
BSS2      YTDNILDDFT YYGKEYVEDK YGG------- LTEAPNNMDT ILDVASEVTF
MS6       YTDNILDDFT YYGKEYVEDK YGG------- LTEAPNNMDT ILDVASEVTF
OS105     YTDNILDDFT YFGKEYVEDK YG-------- ITEAPNNMDT VLDVASEVTF
OS108     YTDNILDDFT YFGKEYVEDK YG-------- ITEAPNNMDT VLDVASEVTF
BSS49     YTDNVLDDFT YFGKEYVEDK YG-------- MTEAPNTMDT VLDVASEVNF
BSS54     YTDNVLDDFT YFGKEYVEDK YG-------- MTEAPNTMDT VLDVASEVNF
OS82      YTDNILDDFT YYGKEYVEDK FG-------- MTEAPNNMDT VLDVGSEVTF
BSS52     YTDNILDDFT SYGVDYVKKK HGA------- LGKVKATQDV VNDIASEVTL
BSS74     YTDNILDDFT SYGVDYVKKK HGA------- LGKVKATQDV VNDIASEVTL
BSS59     YTDNILDDFT SYGVDYVKKK HGA------- LGKVKATQDV VNDIASEVTL
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PL181     YTDNILDDYC YYGLDYVKKN HGG------- LGKAKATQEA VTDIASEVTL
PL1       YTDNILDDYC YYGLDYINSK HGG------- LGKAKKTQEV VNDIATEVTL
PL234     YTDNILDDYC YYGLDYINSK HGG------- IGKAKKTQEV VNDIATEVTL
PL36      YTDNILDDYC YYGLDYIKSK HGG------- IGKAKQTQEV VNDIATEVTL
HL108     YTDNILDDYC YYGLDYIKSK HGG------- LGKAKKTQEV ISDIATEVTL
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PL225     YTDNILDDFC YYGLDYIKSK HGG------- IGKAKATQET VNDIATEVTL
PL43      YTDNILDDYC YYGLDYVKKN HGG------- LGKAKKTQEA INDIATEVTL
PL238     YTDNILDDYC YYGLDYINKN HGG------- LGKAKHTQEV INDIATEVTL
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BSS9      YTDNILDDFT YYGMDYLKDK YGYNYREPGP DRVIKPTQEI VNDLATEVCL
BSD28     YTDNILDEFT YYGMDYIKDK YSVDYTHPSP TDTVRPTQDV VNDIATEVNL
BSD29     YTDNILDEFT YYGMDYIKDK YKVDFKNPSA KDKVKATQDV VNDIATEVTL
BSD63     YTDNILDEFT YYGMDYIKDK YKVDFKNPSA KDKVKATQDV VNDIATEVTL
BSD67     YTDNILDEFT YYGMDYIKDK YKVDWKNPSP KDKVKPTYDI VNDIATEVAL
BSD14     YTDNILDEFT YYGMDYIKDK YKVDWKNPNA NDKVKPTYDV VNDMATEVTL
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BSD21     YTDNILDEFT YYGMDYIKDK YKVDWKNPSA KDKIKPTQDV VNDMATEVTL
BSD95     YTDNILDEFT YYGMDYIKDK YKVDWKNPSA KDKIKPTQDV VNDMATEVTL
BSD61     YTDNILDEFT YYGMDYVKDK YKVDWKNPSP KDKVKPTQEI VNDMATEVTL
BSS46     YTDNILDEFT YYGMDYVKDK YTVDWKNPSP KDKVKPTQEI VNDMATEVTL
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MS61      YTDNILDEFT YYGMDYAKDK YKVDWKNPSP SDKVKPTQEI VNDLATEVTL
PL22      YTDNILDEFT YYGMDYAKDK YGVDWKNPSP NDKVKPTQEI VNDLATEVTL
PL240     YTDNILDEFT YYGMDYAKDK YGVDWRNPSP KDKVKPTQEI VNDLATEVTL
BSD73     YTDNILDEFT YYGMDYIKDK YKVDWKNPSP KDKVKPTYDI VNDIATEVAL
BSD90     YTDNILDEFT YYGMDYVKDK YKVDWKNPSA KDKVKPTYDV VNDMATEVAL
OS15      YTDNILDDFI YHGMDYLHDK YKIDWKNPSP ANNVAATQEV VNDIGTEVNL
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OS59      YTDNILDDFI YHGMDYLHDK YKIDWKNPSP ANNVAATQEV VNDIGTEVNL
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OS41      YTDNILDDFT YYGMDYLHDK YKVDTKNPNA KDKVKATQEV VNDIATEVNL
HL99      YTDNILDDFT YHGMDYLHDK YKIDWKNPNP KDKVKATQEI VNDIATEVNL
OS110     YTDNVLDDFT YYGMDYLHDK YKIDWKNPNP KDKVKATQEV VNDIATEVNL
HL34      YTDNILDDFT YYGMDYLHDK YKIDVKNPNP KDKVKATQEV VSDIATEVNL
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OS63        YTDNILDDFT YYGMDYIHDK YKVDLKNPNP NDKVKATQEV VNDIATEVNL
OS58        YTDNILDDFI YSGMDYIHDK YKIDWKNPNP KDKVKATQEV VNDIATEVNL
OS65        YTDNVLDDFI YSGMDYIHDK YKIDWKNPNP KDKVKATQEV VNDIATEVNL
PL3         YTDNVLDDFI YSGMDYIHDK YKIDWKNPNP KDKVKATQEV VNDIATEVNL
OS27        YTDNILDDFI YSGMDYIHDK HKIDTKNPNP NDKVKATQEV VNDIASEVNL
OS71        YTDNILDDFT YYGMDYLHDK YKIDTKNPNP NDKVKATQEV VNDIASEVNL
PL126       YTDNILDDFV YSGMDYIHDK YNVDLKNPNP NDKVKATQEV VNDIATEVNL
MS16        YTDNILDEFT YYGMDYIKDK YKVDWKACNP ADKVKPTQEV VNDIAGEVTL
MS37        YTDNILDEFT YYGMDYIKDK YKVDWKACNP ADKVKPTQEV VNDIAGEVTL
MS23        YTDNILDEFT YYGMDYIKDK YKVDWKACNP ADKVKPTQEV VNDIAGEVTL
OS48        YTDNILDEFT YYGMDYIKDK YKVDWKACNP ADKVKPTQEV VNDIAGEVTL
PL109       YTDNILDDFT YYGMDYIKSK YKVDWKNPSG KDRVKPTQDI INELATEVTL
PL187       YTDNILDDFT YYGMDYIKSK YKVDWKNPSD KDRVKPTQDL INELATEVTL
PL21        YTDNILDDLT YYGMDYIKNK YKVDWKNPSE KDRVKPTQDL VNELATEVTL
PL206       YTDNILDEFT YYGMDYIKDK YKVNWKSPSD KDKLKPTQDL VNELASEVTL
PL40        YTDNILDEFT YYGMDYIKDK YKVNWKSPSD KDKLKPTQDL VNELAWEVTL
BSS14       YTDNILDDFT YYGMDYIKDK YKVDWKAPSD KDKVKPTQEL VNELASEVTL
BSS43       YTDNILDDFT YYGMDYIKNK YKVDWKSPSG KDKVKPTQEL VNELASEVTL
OS77        YTDNILDEFT YYGMDYLKDK YGG------- YSQAPATQEV VNDLATEVTM
BSS21       YTDDILDNNV YYNVDYINDK YNGAA-NIGT DNKVKATLDV VKDIATESTL
BSS65       YTDDILDNNV YYDVDYINDK YNGAA-NLGT DNKVKATLDV VKDIATESTL
BSS50       YTDDILDNNV YYDVDYINDK YNGAA-NLGT DNKVKATLDV VKDIATESTL
MS22        YTDDILDNNV YYDVDYINDK YNGAA-NLGK DNKVKATLDV VKDIATESTI
MS42        YTDDILDNNV YYDVDYINDK YNGAA-TVGK DNKIKATLEV VKDIATESTI
BSD43       YTNDVLDDFC YYGVDFANDK FGGFA----- --KAPKLLDT AKELATEVNA
BSD79       YTNDVLDDFC YYGVDFANDK FGGFA----- --KAPKLLDT AKELATEVNA
PL53        YTNDVLDDFS YYAADYAVDK FGGFA----- --KAPATVET AKDIATEATL
OS18        YTDNILEDYV YYAIDTIKDK SGGF------ CKLDPNNYDK LMELGDNVNT
OS55        YTDNILEDYV YYAIDTIKDK YGGF------ CKLDPNNYDK LMELGDNVNT
HL74        YTDNILEDYT YYAIDYIKDK YGGF------ CKLDPNNYDE MMKLGYNVNS

            51                                                  100
OS37        YGLEQYD-TP ALLEDHFGGS QRAAVVSAAA GCSVAFATGN SNAGINGWYL
OS70        YGLEQYD-TP ALLEDHFGGS QRAAVVSAAA GCSVAFATGN SNAGINGWYL
BSS22       YGLEQYE-YP ALMEDHFGGS QRTAVVSAAA GCSVAFATGN SNAGINGWYL
BSS26       YGLEQYE-YP ALMEDHFGGS QRTAVVSAAA GCSVAFATGN SNAGINGWYL
MS28        YGLEQYE-YP ALMEDHFGGS QRTAVVSAAA GCSVAFATGN SNAGINGWYL
MS51        YGLEQYE-YP ALMEDHFGGS QRTAVVSAAA GCSVAFATGN SNAGINGWYL
OS102       YGLEQYE-YP ALMEDHFGGS QRTAVVSAAA GCSVAFATGN SNAGINGWYL
OS25        YGLEQYE-YP ALMEDHFGGS QRTAVVSAAA GCSVAFATGN SNAGINGWYL
BSS12       YGLEQYE-YP ALLEDHFGGS QRTAVISAAA GCSCAFATAN SNAGINGWYL
MS19        YGLEQYEEFP ALLEDQFGGS QRAAVVAAAS GCSTGFATGN GQAGLSAWYL
MS26        YGLEQYEEFP ALLEDQFGGS QRAAVVAAAS GCSTGFATGN GQAGLSAWYL
OS111       YGLEQYEEFP ALLEDQFGGS QRAAVVAAAS GCSTGFATGN GQAGLSAWYL
OS61        YGLEQYEEFP ALLEDQFGGS QRAAVVAAAS GCSTGFATGN GQAGLSAWYL
BSS2        YGLEQYEEFP ALLEDQFGGS QRAAVVAAAS GCSTGFATGN GQAGLSAWYL
MS6         YGLEQYEEFP ALLEDQFGGS QRAAVVAAAS GCSTGFATGN GQAGLSAWYL
OS105       YALEQFEDYP ALLETIFGGS QRASIVAAAA GCSTAFATGN AQTGLSGWYL
OS108       YALEQFEDYP ALLETIFGGS QRASIVAAAA GCSTAFATGN AQTGLSGWYL
BSS49       YALEQFEDYP ALLETIFGGS QRASIVAAAS GCSIAFATGN AQTGLSGWYL
BSS54       YALEQFEDYP ALLETIFGGS QRASIVAAAS GCSTAFATGN AQTGLSGWYL
OS82        YALEQFEEYP ALLETIFGGS QRASIVAAAA GCSTGFATGN SQTGLSAWYL
BSS52       YGMEQYEEFP TTLESHFGGS QRATVLAAAS GVSTALATAN SNAGLNGWYM
BSS74       YGMEQYEEFP TTLESHFGGS QRATVLAAAS GVSTALATAN SNAGLNGWYM
BSS59       YGMEQYEEFP TTLESHFGGS QRATVLAAAS GVSTALATAN SNAGLNGWYM
PL145       YGMEQYEQFP TTLESHFGGS QRASVLAAAS GISASLATAN SNAGLNGWYM
PL181       YGMEQYEQFP TTLESHFGGS QRASVLAAAS GISASLATAN SNAGLNGWYM
PL1         YGMEQYEQFP TTLESHFGGS QRASVLAAAS GISCSLATAN SNAGLNGWYM
PL234       YGMEQYEQFP TTLESHFGGS QRASVLAAAS GISCSLATAN SNAGLNGWYM
PL36        YGMEQYEQYP TTLESHFGGS QRASVLAAAS GISCSLATAN SNAGLNGWYM
HL108       YGMEQYEQFP TTLESHFGGS QRASVLAAAS GISCSLATAN SNAGLNGWYM
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PL7       YGMEQYEQFP TTLESHFGGS QRASVLAAAS GISCSLATAN SNAGLNGWYM
PL225     YGMEQYEQFP TTLEDHFGGS QRASVLAAAS GISTSLATAN SNAGLNGWYM
PL43      YGMEQYEQFP TTLESHFGGS QRASVLTAAS GISCSLATAN SNAGLNGWYT
PL238     YGMEQYEQYP TTLESHFGGS QRASVLAAAS GISTSLATAN SNAGLNGWYM
BSS8      YSMEQYEKFP TLMEDHFGGS QRAAVMAAAS GITTAIGTGN SNAGLNAWYL
BSS9      YSMEQYEKFP TLMEDHFGGS QRAAVMAAAS GITTAIGTGN SNAGLNAWYL
BSD28     NGMEQYEQYP TMMEDHFGGS QRAGVLAAAC GLSCSIATGN SNAGLNGWYL
BSD29     NGMEQYEQFP TMMEDHFGGS QRAAVLAAAS GISTAIATGN SNAGLNGWYL
BSD63     NGMEQYEQFP TMMEDHFGGS QRAAVLAAAS GISTAIATAN SNAGLNGWYL
BSD67     NGMEQYEQYP TMMEDHFGGS QRAGVIAAAS GLTTAIATGN SNAGLNGWYL
BSD14     NAMEQYEQFP TMMEDHFGGS QRAGVIAAAS GLTTAIATGN SNAGLNGWYL
BSD42     NAMEQYEQFP TMMEDHFGGS QRAGVIAAAS GLTTSIATGN SNAGLNAWYL
BSD21     NAMEQYEQFP TMMEDHFGGS QRAGVIAAAS GLTTAIATGN SNAGLNGWYL
BSD95     NAMEQYEQFP TMMEDHFGGS QRAGVIAAAS GLTTAIATGN SNAGLNGWYL
BSD61     NAMEQYEMFP TMMEDHFGGS QRAGVIAAAS GLTTSIATGN SNAGLNAWYL
BSS46     NAMEQYEMFP TMMEDHFGGS QRAGVIAAAS GLTTAIATGN SNAGLNGWYL
HL81      NAMEQYEQFP TMMEDHFGGS QRAGVIAAAS GLTTSITTGN SNAGLNGWYL
MS61      NAMEQYEQYP TMMEDHFGGS QRAGVIAAAS GLTTAIATGN SNAGLNAWYL
PL22      NAMEQYEQFP TMMEDHLGGS QRAGVIAAAS GLTTFITTGN SNAGLNAWYL
PL240     NAMEQYEQFP TMMEDHFGGS QRAGVIAAAS GLTTSITTGN SNAGLNAWYL
BSD73     NGMEQYEQYP TMMEDHFGGS QRAGVLAAAC GLSTSIATGN SNAGLNAWYL
BSD90     NGMEQYEQYP TMMEDHFGGS QRAGVLAAAC GLSTSIATGN SNAGLNAWYL
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OS20      YGMEQYEQFP TMLEDHFGGS QRASVLAAAS GITTSIATGN SNAGLNAWYL
OS59      YGMEQYEQFP TMLEDHFGGS QRASVLAAAS GITTSIATGN SNAGLNAWYL
OS80      YGMEQYEQFP TMLEDHFGGS QRASVLAAAS GITTSIATGN SNAGLNAWYL
HL110     YGMEQYEQFP TMMEDHFGGS QRAAVLAAAS GISTSIATGN SNAGLNGWYL
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HL99      YGMEQYEQFP TMLEDHFGGS QRAAVLGAAC GLTTSIATGN SNAGLNGWYL
OS110     YGMEQYEQFP TMMEDHFGGS QRAAVLAAAS GITTSIATGN SNAGLNGWYL
HL34      YGMEQYEQFP TMLEDHFGGS QRAAVLGAAS GITTSIATGN SNAGLNGWYL
OS63      YGMEQYEQFP TMLEDHFGGS QRAAVLGAAC GLTTSIATGN SNAGLNGWYL
OS58      YGMEQYEQFP TMMEDHFGGS QRASVLAAAS GITTSIATSN SNAGLNAWYL
OS65      YGMEQYEQFP TMMEDHFGGS QRASVLAAAS GITTSIATAN SNAGLNAWYL
PL3       YGMEQYEQFP TMMEDHFGGS QRASVLAAAS GITTSIATAN SNAGLNGWYL
OS27      YGMEQYEQFP TMMEDHFGGS QRAAVLAAAS GITTSIATSN SNAGLNAWYL
OS71      YGMEQYEQFP TMMEDHFGGS QRAAVLAAAS GITTSIATSN SNAGLNGWYL
PL126     YGMEQYEQFP TMMEDHFGGS QRAAVLAAAS GITTSITTGN SNAGLNGWYL
MS16      NAMEQYEQFP TLMEDHFGGS QRAGVIAAAS GLSTAIATAN SNAGLNGWYL
MS37      NAMEQYEQFP TLMEDHFGGS QRAGVIAAAS GLSTAIATAN SNAGLNGWYL
MS23      NAMEQYEQFP TLMEDHFGGS QRAGVIAAAS GLSTAIATAN SNAGLNGWYL
OS48      NAMEQYEQFP TLMEDHFGGS QRAAVLAAAS GITTSIATGN SNAGLNGWYL
PL109     YGMEQYEEFP TTLESHFGGS QRAGVLAAAS GLTCAIATAN SNAGLNGWYM
PL187     YGMEQYEEFP TTLESHFGGS QRAGVLAAAS GLTCAIATAN SNAGLNGWYM
PL21      YGMEQYEEFP TTLESHFGGS QRAGVLAAAS GLTCAIATAN SNAGLNGWYM
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PL40      YGMEQYEQFP TMMEDHFGGS QRAGVLAAAA GLTTSIATAN SNAGLNGWYM
BSS14     YGMEQYEQFP TMMEDHFGGS QRAGVLAAAS GLTTAIATGN SNAGLNGWYM
BSS43     YGMEQYEQFP TMMEDHFGGS QRAGVLAAAS GLTTAIATGN SNAGLNGWYM
OS77      NAMEQYEQFP TMMEDHFGGS QRAGVIAAAS GLTTAIGTGN SNAGLNGWYL
BSS21     YGIETYEKFP TALEGHFGGS QRATVLAAAA GVATALATAN ANAGLSGWYL
BSS65     YGIETYEKFP TALEDHFGGS QRATVLAAAA GVACALGTAN ANAGLSGWYL
BSS50     YGIETYEKFP TALEDHFGGS QRATVLAAAA GVACALGTVN ANAGLSGWYL
MS22      YGIETYEKFP TALEDHFGGS QRATVLAAAA GVAVALGTAN ANAGLSGWYL
MS42      YGIETYEKFP TALEDHFGGS QRATVLAAAA GVAVALGTAN ANAGLSGWYL
BSD43     YGMEQYEAFP TLLEDHFGGS QRASVLAAAS GITSAIASGH SQVGLAGWYL
BSD79     YGMEQYEAFP TLLEDHFGGS QRASVLAAAS GITSAIASGH SQVGLAGWYL
PL53      YGIEQYESFP TLVEDHFGGS QRASVLAACS GIGCGLATGH SQIGLAGWYL
OS18      YALEMYERYP AAMEAHFGGS QRATVAAAAS GIAGSMATGN ADCGVNMWYL
OS55      YALEMYEKYP AVMEAHFGGS QRATVAAAAS GIAGAMATGN ADCGVNMWYL
HL74      YALETYEKYP AAMETHFGGS QRATVAAAST GIAGSMATGI ADCGLNLWYL
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          101                                                  146
OS37      SQILHKEAHS RLGFYGYDLQ DQCGASNSLS IRSDEGLIHE LRGPNY
OS70      SQILHKEAHS RLGFYGYDLQ DQCGASNSLS IRSDEGLIHE LRGPNY
BSS22     SQILHKEAHS RLGFYGYDLQ DQCGASNSLS IRSDEGLIHE LRGPNY
BSS26     SQILHKEAHS RLGFYGYDLQ DQCGASNSLS IRSDEGLIHE LRGPNY
MS28      SQILHKEAHS RLGFYGYDLQ DQCGASNSLS IRSDEGLIHE LRGPNY
MS51      SQILHKEAHS RLGFYGYDLH DQCGASNSLS IRSDEGLIHE LRGPNY
OS102     SQILHKEAHS RLGFYGYDLQ DQCGASNSLS IRSDEGLIHE LRGPNY
OS25      SQILHKEAHS RLGFYGYDLQ DQCGASNPLS IRSDEGLIHE LCGPNY
BSS12     SQILHKEGHS RLGFYGYDLQ DQCGASNSLS IRSDEGLIHE LRGANY
MS19      SMYLHKEQHS RLGFYGYDLQ DQCGASNVFS IRGDEGLPLE LRGANY
MS26      SMYLHKEQHS RLGFYGYDLQ DQCGASNVFS IRGDEGLPLE LRGANY
OS111     SMYLHKEQHS RLGFYGYDLQ DQCGASNVFS IRGDEGLPLE LRGANY
OS61      SMYLHKEQHS RLGFYGYDLQ DQCGASNVFS IRGDEGLPLE LRGANY
BSS2      SMYLHKEQHS RLGFYGYDLQ DQCGASNVYS IRGDEGLPLE LRGANY
MS6       SMYLHKEQHS RLGFYGYDLQ DQCGASNVFS IRGDEGLPLE LRRANY
OS105     SMYLHKEQHS RLGFYGYDLQ DQCGASNVFS IRGDEGLPSE LRGANY
OS108     SMYLHKEQHS RLGFYGYDLQ DQCGASNVFS IRGDEGLPTE LRGANY
BSS49     SMYLHKEQHS RLGFYGYDLQ DQCGASNVFS IRGDEGLPTE LRGANY
BSS54     SMYLHKEQHS RLGFYGYDLQ DQCGASNVFS IRGDEGLPTE LRGANY
OS82      SMYLHKEQHS RLGFYGYDLQ DQCGASNVFS IRNDEGLPVE MRGPNY
BSS52     SMLMHKEAWS RLGFFGYDLQ DQCGSANSMS VRPDEGLLGE LRGPNY
BSS74     SMLMHKEAWS RLGFFGYDLQ DQCGSANSMS VRPDEGLLGE LRGPNY
BSS59     SMLMHKEAWS RLGFFGYDLQ DQCGSANSTS VRPDEGLLGE LRGPNY
PL145     SMLMHKEGWS RLGFFGYDLQ DQCGSANSLS VRPDEGCVGE LRGPNY
PL181     SMLMHKEGWS RLGFFGYDLQ DQCGSANCMS VRPDEGCIGE LRGPNY
PL1       SMLAHKEGWS RLGFFGYDLQ DQCGSANSMS VRPDEGCMGE LRGPNY
PL234     SMLAHKEGWS RLGFFGYDLQ DQCGSANSMS VRPDEGCIGE LRGPNY
PL36      SMLAHKEGWS RLGFFGYDLQ DQCGSANSMS IRPDEGCIGE LRGPNY
HL108     SMLAHKEGWS RLGFFGYDLQ DQCGSTNSMS IRPDEGCIGE LRGPNY
PL7       SMLAHKEGWS RLGFFGYDLQ DQCGSANSMA IRPDEGCIGE LRGPNY
PL225     SMLAHKEGWS RFGFFGYDLQ DQCGSANSLS VRPDEGCVGE LRGPNY
PL43      SMLAHKEGWP RFGFFGYDLQ DQCGSSNSLS VRPDEGCVGE LRGPNY
PL238     SMLAPKEGWS RLGFFGYDLQ DQCGSANCMA IRPDEGCIGE LRGPNY
BSS8      AMIIHKDAWS RLGFFGYDLQ DQCGSANSLS IRGDQGAIRE GRGPNY
BSS9      AMIIHKDAWS RLGFFGYDLQ DQCGSANSLS MEPDRGAMGE LRGPNY
BSD28     SMLMHKEGWS RLGFFGYDLQ DQCGSANSLS MEPDRGLMGE LPGPNY
BSD29     SMLLHNDGWS RLGFFGYDLQ DQCGSANSLS MESDRGLMGE LPGPNY
BSD63     SMLLHKEGWS RLGFFGYDLQ DQCGSTNSLS VRPDEGCIGE FRGPNY
BSD67     SMLLHKDGWS RLGFFGYDLQ DQCGSANSLS IRGDQGAIRE VRGPNY
BSD14     SMLLHKDGWS RLGFFGYELQ DQCGSANSLS IRGDQGAIRE LPGPNY
BSD42     SMLLHKDGWS RLGFFGYDLQ DQCGSANSLS MESDRGLIGE LPGPNY
BSD21     SMLLHKDGWS RLGFFGYDLH DQCGSANSLS MGADRGLIRE LPGPNY
BSD95     SMLLHKDGWS RLGFFGYDLQ DQCGSANSLS MEPDRGLMGE LRGPNY
BSD61     CMLLHKDGWS RLGFFGYDLQ DQCGSANSLS MESDRGLMGE LRGPNY
BSS46     SMLLHKDGWS RLGFFGYDLQ DQCGSANSLS MEPDRGLMGE LRGPNY
HL81      SMLLHKDGWS RLGFFGYDLQ DQCGSANSLS MESDRGLMGE LRGPNY
MS61      SMLLHKDGWS RLGFFGYDLQ DQCGSANSLS MESDRGLMGE LRGPNY
PL22      SMLLHKDGWS RLGFFGYDLQ DQCGSANSLS MESDRGLMGE LRGPNY
PL240     SMLLHKDGWS RLGFFGYDLQ DQCGSANSLS MESDRGLVGE LRGPNY
BSD73     SMLLHKDGWS RLGFFGYDLQ DQCGSANSLS MEPDRGLMGE LRGPNY
BSD90     SMLLHKDGWS RLGFFGYDLQ DQCGSANSLS MESDRGLMGE LRGPNY
OS15      SMLMHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
OS20      SMLMHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
OS59      SMLMHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
OS80      SMLMHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
HL110     SMLLHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
OS41      SMLLHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
HL99      SMLMHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
OS110     SMLLHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
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HL34        SMLMHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
OS63        SMLMHKDGWS RLGFSGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
OS58        SMLMHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
OS65        SMLMHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
PL3         SMLMHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGG FRGPNY
OS27        SMLMHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
OS71        SMLMHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
PL126       SMLLHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
MS16        SMLMHKEGWS RLGFFGYDLQ DQCGSTNSLS VRPDEGCIGE YRGPNY
MS37        SMLMHKEGWS RLGFFGYDLQ DQCGSTNSLS VRPDEGCIGE YRGPNY
MS23        SMLMHKEGWS RLGLFGYDLQ DQCGSTNSLS VRPDEGCIGE YRGPNY
OS48        SMLLHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
PL109       SMLAHKEGWS RLGFFGYDLQ DQCGSANCMA IRPDEGCIGE LRGPNY
PL187       SMLAHKEGWS RLGFFGYDLQ DQCGSANCMA IRPDEGCIGE LRGPNY
PL21        SMLAHKEGWS RLGFFGYDLQ DQCGSANCMA IRPDEGCVGE LRGPNY
PL206       SMLLHKEGWS RLGFFGYDLQ DQCGSANSLS VRPDEGCVGE LRGPNY
PL40        SMLLHKEGWS RLGFFGYDLQ DQCGSANSLS VRPDEGCVGE LRGPNY
BSS14       SMLMHKEGWS RLGFFGYDLQ DQCGSANSLS VRPDEGCVGE LRGPNY
BSS43       SMLLHKEGWS RLGFFGYDLQ DQCGSANSLS VRPDEGCVGE LRGPNY
OS77        SMLMHKEGWS RLGFFGYDLQ DQCGSANSMS IRPDEGLLGE LRGPNY
BSS21       SMYLHKEAWG RLGFFGYDLQ DQCGATNVLS YQGDEGLPDE LRGPNY
BSS65       SMYLHKEAWC RLGFFGFDLQ DQCGATNVLS YQGDEGLPDE LRGPNY
BSS50       SMYLHKEAWG RLGFFGFDLQ DQCGATNVLS YQGDEGLPDE LRGPNY
MS22        SMYLHKEAWG RLGFFGYDLQ DQCGATNVLS YQGDEGLPDE LRGPNY
MS42        SMYLHKEAWG RLGFLGYDLQ DQCGATNVLS YQGDEGLPDE LRGPNY
BSD43       SMLLHKEGWG RLGFFGYDLQ DQCGPTNVFS YQSDEGNPLE LRGANY
BSD79       SMLLHKEGWG RLGFFGYDLQ DQCGPTNVFS YQSDEGNPLE LRGANY
PL53        SMLLHKEAWG RLGFFGYDLQ DQCGPTNVFS YQSDESNPLE LRGANY
OS18        SMLQHKERTG RLGFYGYDLQ DQCGSANSFS YRSDEGLPME LRGPNY
OS55        SMLQHKERTG RLGFYGYDLQ DQCGSANSFS YRSDEGLPME LRGPNY
HL74        SMLQQKERTG RLGFYGYDLQ DQCGSANSFA YRSDEGLPME LRGPNY
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APPENDIX C.

Alignment of nucleotide sequences determined in this study for *mcrA* PCR products from described methanogen species.

Methanogen species used for *mcrA* sequences determination:

1. *Methanobacterium formicicum* (DSM 1312)
2. *Methanobrevibacter arboriphilicus* (DSM 1125)
3. *Methanobrevibacter ruminantium* (DSM 1093)
4. *Methanoculleus bourgensis* (DSM 3045)
5. *Methanosprillum hungatei* (DSM 864)
6. *Methanocorpusculum aggregans* (DSM 3027)
7. *Methanocorpusculum bavaricum* (DSM 4179)
8. *Methanocorpusculum parvum* (DSM 3823)
9. *Methanosarcina mazei* (DSM 2053)
10. *Methanohalophilus halophilus* (DSM 3094)
11. *Methanosaeta concilii* (DSM 3671)
12. *Methanopyrus kandleri* (DSM 6324)

The alignment was created using PILEUP (GCG Wisconsin Package 10.1, Genetics Computer Group, Wisconsin, USA).

Gap creation penalty: 1

Gap extension penalty: 1

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          1                                                   50

M.for.    TACACTGACA ATATCCTGGA TGACTTCGTA TACTACGGAA TGGAATACGT

M.arb.    TACACTGACA ATATTCTGGA CGATTTCTTA TATTATGGTA AAGAATATGT
M.rum.    TACACCGATA ACGTATTAGA CGACTTCTCT TACTTCGGTA AAGATTACGT
M.bou.    TACACCGACA ACATCCTCGA TGAGTTCACC TACTACGGTA TGGACTACAT
M.hun.    TACACCGACA ACATCCTCGA TGAGTTCACC TACTATGGTA TGGACTACAT
M.agg.    TACACGGATA ACATCCTTGA TGACTTCACC TACTACGGAA TGGACTACCT
M.bav.    TACACGGATA ACATCCTTGA TGACTTCACC TACTACGGAA TGGACTACAT
M.par.    TACACGGATA ACATCCTTGA TGACTTCACC TACTACGGAA TGGACTACAT
M.maz.    TACACAGACG ACATCCTCGA CAACAACACC TACTATGACG TTGACTACAT
M.hal.    TACACCAACA ACATTCTGGA TGACAACCTG TACTATGACG TTGAATACAT
M.con.    TACACCAACG ATGTCCTGGA TGACTTCTCC TATTATGGTG TGGACTACGC
M.kan.    TACACTGATA ACATCCTGGA CGACTACGTG TACTACGGTC TCGAGTACGT

          51                                                  100
M.for.    GGACGACAAA TAC..GGT.. .ATTTGTG.. .......... ..........
M.arb.    CGAAGACAAA TTT..GGA.. .ATATGTGAA .......... .........G
M.rum.    AGAAGACAAA TAC..GGAGA CTTATGTTCC .......... .........G
M.bou.    CAAGGACAAG TACAAGGTCG ACTGGAAGAA CCCGAGCGCG AAGGACAAGG
M.hun.    CAAGGACAAG TACAAAGTCG ACTGGAAGAA CCCAAGCCCG AAAGACAAGG
M.agg.    CCACGACAAG TACAAGGTCG ACACCAAGAA TCCGAACGCA AAAGACAAAG
M.bav.    CCACGACAAG TACAAGGTCG ACACCAAGAA TCCGAACGCA AAAGACAAAG
M.par.    CCACGACAAG TACAAGGTCG ACACCAAGAA TCCGAACGCA AAAGACAAAG
M.maz.    CAACGACAAG TACAACGGTG .CTGCAA..A CCTCGGAACT GACAACAAGG
M.hal.    CAACGACAAG TATGACGGTG .CAGCTG..A CAAAGGTATC GACAACAAGG
M.con.    CAACGACAAG TTCGGCGGAT TCGCCAAG.. ...GCACCCG CGACCA....
M.kan.    CGAGGACAAG TAC..GG... AATCGCCGAG .......... .........G

          101                                                 150
M.for.    GAACCAAAGC AACCACTGAA GTGGTTCACG ACATAGCCTC AGAAGTAACC
M.arb.    CTCCAAACAA CATGGACACT GTTCTTGATG TAGGTTCTGA AGTAACTTTC
M.rum.    CACCTAACGA CATGGACACC GTTCTTGACG TAGGTTCTGC AGTAACATTC
M.bou.    TCAAGCCGAC CCAGGATGTC GTCAACGACA TGGCAACCGA GGTCACCCTC
M.hun.    TCAAGCCAAC CCAGGAGATC GTCAACGACA TTGCCGGAGA GGTCACCCTC
M.agg.    TCAAAGCAAC CCAGGAAGTT GTCAACGACA TCGCATCCGA AGTCAACCTT
M.bav.    TCAAAGCAAC GCAGGAAGTT GTCAACGACA TTGCAACCGA AGTCAACCTT
M.par.    TCAAAGCAAC CCAGGAAGTT GTCAACGACA TCGCAACCGA AGTCAACCTT
M.maz.    TAAAGGCAAC CCTCGATGTA GTAAAAGACA TCGCAACCGA GTCCACACTC
M.hal.    TCGCCCCAAG CATGGATGTT ATCAAGGATA TCGCAACAGA GTCCACACTC
M.con.    .......... .T.CGATGTC GCCAAGGAGC TGGCCACTGA GGTCACTCTG
M.kan.    CCGAGCCGAG CATGGACGTG GTGAAGGACG TCGCGACCGA GGTCACCCTG

          151                                                 200
M.for.    ATGTCCGGAC TGGAACAGTA CGAATACCCA GCACTCATGG AAGACCA.CT
M.arb.    TATGGATTAG AACAATACGA AGAATATCCT GCATTACTTG AAACCCA.AT
M.rum.    TACTCATTAG AACAATACGA AGAATACCCA GCTTTACTTG AAACTCA.CT
M.bou.    AACGCCATGG AGCAGTACGA GCAGTTCCCG ACCATGATGG AAGACCA.CT
M.hun.    AATGCAATGG AGCAGTACGA ACAGTTCCCA ACCATGATGG AAGACCA.CT
M.agg.    TACGGTATGG AGCAGTATGA ACAGTTCCCG ACCATGATGG AAGACCA.CT
M.bav.    TACGGTATGG AACAGTATGA ACAGTTCCCG ACCATGATGG AAGACCA.CT
M.par.    TACGGTATGG AACAGTATGA ACAGTTCCCG ACCATGATGG AAGACCA.CT
M.maz.    TATGGTATCG AGACCTACGA GAAATTCCCA ACAGCCCTTG AAGACCA.CT
M.hal.    TACGGTCTCG AGAACTACGA GAAATACCCA GTTGCACTTG AAGACCA.CT
M.con.    TACGGCATTG AGCAGTACGA GGCCTTCCCC ACCCTGCTTG AGGATCA.CT
M.kan.    TACGGTCTGG AGCAGTACGA GCGGTACCCG GCCGCCATGG AGACGCA.CT

          201                                                 250
M.for.    TCGGTGGATC CCAGAGGACT GCAGTTGTTT CTGCTGCTGC CGGATGTTCC
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M.arb.  TCGGTGGTTC TCAAAGAGCA TCTGTTGTTT CAGCCGCTGC TGGTTGTGCA
M.rum.  TCGGTGGTTC TCAAAGAGCT GCTGTTGTAT CTGCAGCTTC AGGTATTTCC
M.bou.  TCGGCGGGTC CCAGCGTGCA GGTGTCATCG CCGCTGCGTC CGGTCTCACG
M.hun.  TTGGTGGTTC CCAGCGTGCA GGAGTTATCG CAGCAGCATC CGGTCTGTCT
M.agg.  TCGGCGGTTC CCAGCGTGCA GCCGTTCTTG CAGCAGCCTC CGGTATCTCC
M.bav.  TCGGTGGTTC CCAGCGTGCA TCCGTTCTTG CAGCAGCATC CGGTATCACC
M.par.  TCGGCGGTTC CCAGCGTGCA GCCGTTCTTG CAGCAGCCTC CGGTATCTCC
M.maz.  TCGGTGGGTC CCAGAGAGCA ACCGTGCTTG CAGCTGCATC AGGCGTTGCA
M.hal.  TCGGTGGATC CCAGAGAGCA ACCGTCCTGT CCGCAGCCGC CGGTAGTGCA
M.con.  TCGGCGGGTC CCAGAGGGCA GCCGTTCTGG CAGCCGCTTC CGGTATCACC
M.kan.  TCGGAGGTTC ACAGCGAGCC GCCGTCTGTG CGGCCGCCGC GGGCTGCAGT

        251                                                 300
M.for.  GTTGCCTTTG CAACCGGAAA CTCCAACGCA GGAATTAACG GATGGTACTT
M.arb.  ACAGCTTTCG CTACTGGAAA TTCCCAAACT GGTTTAAGCG CATGGTATTT
M.rum.  ACTGCATTCG CAACTGGAAA CGCACAAACC GGTTTATCTG CATGGTACTT
M.bou.  ACCGCCATCG CAACCGGCAA CTCCAACGCC GGTCTCAACG GATGGTATCT
M.hun.  GTCGGTGTCG CAACAGCAAA CTCCAACGCA GGTCTGAACG GATGGTACCT
M.agg.  ACGTCGATTG CAACCGGAAA CTCCAACGCC GGTCTCAACG GCTGGTATCT
M.bav.  ACGTCAATTG CAACCGGAAA CTCCAACGCC GGTCTCAACG CCTGGTATCT
M.par.  ACGTCGATTG CAACCGGAAA CTCCAACGCC GGTCTCAACG GCTGGTATCT
M.maz.  TGCGCTCTTG CAACCGGAAA CGCAAATGCT GGTCTCTCAG GCTGGTACCT
M.hal.  GGATCCCTTG CAACCGGTAA CGCAAACGCC GGTCTCTCCG CATGGTATCT
M.con.  TCCGCCATCG CCACTGGTCA CTCCCAGATC GGCCTGGCCG GCTGGTATCT
M.kan.  ACCGCCTTCG CGACCGGTCA CGCGCAGGCA GGACTCAACG GTTGGTACCT

        301                                                 350
M.for.  AAGCCAGATC CTGCAC.AAA GAAGCACACA GCAGACTCGG TTTCTACGGT
M.arb.  ATCTATGTAC TTACAC.AAA GAACAACACT CTAGATTAGG ATTCTATGGT
M.rum.  AGCACAATAC TTACAC.AAA GAACAACATT CCAGATTAGG ATTCTACGGT
M.bou.  CTCGATGCTC CTGCAC.AAG GACGGCTGGT CGCGTCTCGG CTTCTTCGGC
M.hun.  CTCCATGCTC ATGCAC.AAG GAAGGCTGGT CACGTCTCGG ATTCTTCGGA
M.agg.  GTCCATGCTT CTGCAC.AAA GACGGCTGGT CCAGACTTGG TTTCTTCGGC
M.bav.  GTCCATGCTT ATGCAC.AAA GACGGCTGGT CCAGACTTGG TTTCTTCGGC
M.par.  GTCCATGCTT CTGCAC.AAA GACGGCTGGT CCAGACTTGG TTTCTTCGGC
M.maz.  CTCCATGTAT GTCCAC.AAG GAAGCATGGG GCAAACTCGG ATTCTTCGGT
M.hal.  CTGTATGTAC CTGCAC.AAG GAAGGTCACG GACGTCTCGG ATTCTTCGGA
M.con.  CTCCATGCTC CTGCAC.AAG GAAGCCTGGG GCAGACTGGG ATTCTTCGGC
M.kan.  GTCGCAGATC CTGCAC.AAG GAGGGTCACG GTCGTCTAGG ATTCTACGGG

        351                                                 400
M.for.  TACGACCTGC AGGACCAGTG TGGAGCATCC AACTCTCTCT CCATCAGGAG
M.arb.  TACGATTTAC AAGATCAATG TGGTGCATCC AACGTATTCT CTATAAGAAA
M.rum.  TACGACTTGC AAGATCAATG TGGTGCAGCT AACGTATTCG CAATCAGAAA
M.bou.  TACGACCTCC AGGACCAGTG CGGGTCTGCA AACTCGCTCT CCATGGAGCC
M.hun.  TACGACCTGC AGGACCAGTG TGGTTCCACC AACTCACTCT CTGTCAGACC
M.agg.  TACGATCTGC AGGACCAGTG CGGTTCCGCA AACTCACTCT CCATCAGACC
M.bav.  TACGATCTGC AGGACCAGTG CGGTTCCGCA AACTCACTCT CCATCAGACC
M.par.  TACGATCTGC AGGACCAGTG CGGTTCCGCA AACTCACTCT CCATCAGACC
M.maz.  TTCGACCTGC AGGATCAGTG TGGTGCCACA AACGTTCTGT CCTACCAGGG
M.hal.  TTCGACCTGC AGGACCAGTG TGGTGCAACC AACACCTTCT CCTACCAGTC
M.con.  TACGACCTGC AGGATCAGTG CGGTCCAACC AATGTGTTCT CTTACCAGTC
M.kan.  TACGCCCTAC AGGACCAGTG TGGTGCGGCC AACTCGCTGA GCGTGAGGAG

        401                                         440
M.for.  CGACGAAGGT TTAATCCACG AACTACGTGG TCCTAACTAC
M.arb.  TGACGAAGGA TTACCAGTTG AAATGAGAGG GCCAAACTAC
M.rum.  CGACGAAGGT TTACCACTTG AATTAAGAGG ACCTAACTAC
M.bou.  CGACCACGGT CTTATCGGCG AGCTGCGTGG TCCGAACTAC
M.hun.  TGACGAGGGT TGTATCGGTG AATACCGTGG TCCAAACTAC
M.agg.  TGATGAAGGC TGTATCGGCG AGTTCCGCGG ACCAAACTAC
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M.bav.  TGATGAAGGC TGTATCGGCG AGTTCCGCGG ACCAAACTAC
M.par.  TGATGAAGGC TGTATCGGCG AGTTCCGCGG ACCAAACTAC
M.maz.  CGACGAAGGT CTCCCAAACA AACTCCGTGG TCCAAACTAC
M.hal.  CGATGAAGGT CTGCCCCACG AACTCCGTGG TCCAAACTAT
M.con.  AGACGAGGGC AACCCATTGG AGCTGAGAGG CGCCAACTAC
M.kan.  CGACGAGGGA CTGCCGCTCG AGCTGCGTGG TCCGAACTAC
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APPENDIX D.

Alignment of predicted amino acid sequences determined in this study for *mcrA* PCR products from described methanogen species.

Methanogen species used for *mcrA* sequences determination:

13. *Methanobacterium formicicum* (DSM 1312)
14. *Methanobrevibacter arboriphilicus* (DSM 1125)
15. *Methanobrevibacter ruminantium* (DSM 1093)
16. *Methanoculleus bourgensis* (DSM 3045)
17. *Methanosprillum hungatei* (DSM 864)
18. *Methanocorpusculum aggregans* (DSM 3027)
19. *Methanocorpusculum bavaricum* (DSM 4179)
20. *Methanocorpusculum parvum* (DSM 3823)
21. *Methanosarcina mazei* (DSM 2053)
22. *Methanohalophilus halophilus* (DSM 3094)
23. *Methanosaeta concilii* (DSM 3671)
24. *Methanopyrus kandleri* (DSM 6324)

The alignment was created using PILEUP (GCG Wisconsin Package 10.1, Genetics Computer Group, Wisconsin, USA).

Gap creation penalty: 8

Gap extension penalty: 2

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1.  M. formicicum          YTDDILDDFV YYGMEYVDDK YG-------- ICGTKATTEV
2.  M. arboriphilicus      YTDNILDDFL YYGKEYVEDK FG-------- ICEAPNNMDT
3.  M. ruminantium         YTDNVLDDFS YFGKDYVEDK YGD------- LCSAPNDMDT
4.  M. bourgensis          YTDNILDEFT YYGMDYIKDK YKVDWKNPSA KDKVKPTQDV
5.  M. hungatei            YTDNILDEFT YYGMDYIKDK YKVDWKNPSP KDKVKPTQEI
6.  M. aggregans           YTDNILDDFT YYGMDYLHDK YKVDTKNPNA KDKVKATQEV
7.  M. bavaricum           YTDNILDDFT YYGMDYIHDK YKVDTKNPNA KDKVKATQEV
8.  M. parvum              YTDNILDDFT YYGMDYIHDK YKVDTKNPNA KDKVKATQEV
9.  M. mazei               YTDDILDNNT YYDVDYINDK YNGAA-NLGT DNKVKATLDV
10. M. halophilus          YTNNILDDNL YYDVEYINDK YDGAA-DKGI DNKVAPSMDV
11. M. concilii            YTNDVLDDFS YYGVDYANDK FGGFA----- --KAPATIDV
12. M. kandleri            YTDNILDDYV YYGLEYVEDK YG-------- IAEAEPSMDV

1.  VHDIASEVTM SGLEQYE-YP ALMEDHFGGS QRTAVVSAAA GCSVAFATGN SNAGINGWYL
2.  VLDVGSEVTF YGLEQYEEYP ALLETQFGGS QRASVVSAAA GCATAFATGN SQTGLSAWYL
3.  VLDVGSAVTF YSLEQYEEYP ALLETHFGGS QRAAVVSAAS GISTAFATGN AQTGLSAWYL
4.  VNDMATEVTL NAMEQYEQFP TMMEDHFGGS QRAGVIAAAS GLTTAIATGN SNAGLNGWYL
5.  VNDIAGEVTL NAMEQYEQFP TMMEDHFGGS QRAGVIAAAS GLSVGVATAN SNAGLNGWYL
6.  VNDIASEVNL YGMEQYEQFP TMMEDHFGGS QRAAVLAAAS GISTSIATGN SNAGLNGWYL
7.  VNDIATEVNL YGMEQYEQFP TMMEDHFGGS QRASVLAAAS GITTSIATGN SNAGLNAWYL
8.  VNDIATEVNL YGMEQYEQFP TMMEDHFGGS QRAAVLAAAS GISTSIATGN SNAGLNGWYL
9.  VKDIATESTL YGIETYEKFP TALEDHFGGS QRATVLAAAS GVACALATGN ANAGLSGWYL
10. IKDIATESTL YGLENYEKYP VALEDHFGGS QRATVLSAAA GSAGSLATGN ANAGLSAWYL
11. AKELATEVTL YGIEQYEAFP TLLEDHFGGS QRAAVLAAAS GITSAIATGH SQIGLAGWYL
12. VKDVATEVTL YGLEQYERYP AAMETHFGGS QRAAVCAAAA GCSTAFATGH AQAGLNGWYL

1.  SQILHKEAHS RLGFYGYDLQ DQCGASNSLS IRSDEGLIHE LRGPNY
2.  SMYLHKEQHS RLGFYGYDLQ DQCGASNVFS IRNDEGLPVE MRGPNY
3.  AQYLHKEQHS RLGFYGYDLQ DQCGAANVFA IRNDEGLPLE LRGPNY
4.  SMLLHKDGWS RLGFFGYDLQ DQCGSANSLS MEPDHGLIGE LRGPNY
5.  SMLMHKEGWS RLGFFGYDLQ DQCGSTNSLS VRPDEGCIGE YRGPNY
6.  SMLLHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
7.  SMLMHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
8.  SMLLHKDGWS RLGFFGYDLQ DQCGSANSLS IRPDEGCIGE FRGPNY
9.  SMYVHKEAWG KLGFFGFDLQ DQCGATNVLS YQGDEGLPNK LRGPNY
10. CMYLHKEGHG RLGFFGFDLQ DQCGATNTFS YQSDEGLPHE LRGPNY
11. SMLLHKEAWG RLGFFGYDLQ DQCGPTNVFS YQSDEGNPLE LRGANY
12. SQILHKEGHG RLGFYGYALQ DQCGAANSLS VRSDEGLPLE LRGPNY
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